

1 **PHENYL XANTHENE DYES**

2 **1. FIELD OF THE INVENTION**

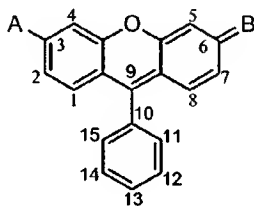
3 The invention relates to fluorescent phenyl xanthene dyes. More specifically, the
4 invention relates to rhodamines, fluoresceins and rhodols.

5 **2. BACKGROUND OF THE INVENTION**

6 There is an ever present need to develop improved fluorescent dyes, especially dyes that
7 exhibit enhanced fluorescence and enhanced stability. In addition, there is a need to develop
8 dyes that can be employed, if desired, in polymeric beads or particles. These and other needs are
9 met by the various dyes described herein.

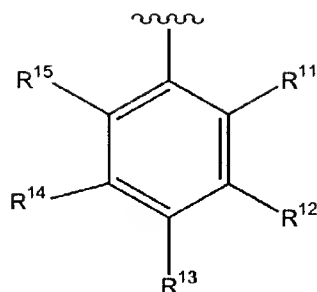
10 **3. SUMMARY OF THE INVENTION**

11 In one aspect, the present invention provides phenyl xanthene dyes that exhibit useful
12 fluorescent properties. The phenyl xanthene dyes comprise any fluorescein, rhodol or
13 rhodamine ring where the phenyl substituent at the 9-carbon ("the C9 phenyl ring") is a specific
14 type of phenyl ring. For the purposes of this description, fluoresceins, rhodols and rhodamines
15 are numbered in the following manner:



16 where A is either a hydroxyl or an amine group and B is either an oxo or an imminium group.
17 The C9 phenyl ring on the phenyl xanthene dye, whether substituted or unsubstituted, may be
18 referred to as the "lower ring." The remainder of the molecule may be referred to as the "upper
19 ring."
20 ring."

21 The C9 phenyl ring is substituted at one or both of carbons C11 or C15 with a group
22 selected from alkyl, heteroalkyl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano, isocyano,
23 cyanato, mercaptocyanato, nitro, and sulfinyl. When both the C-11 and C-15 carbons are
24 substituted, the substituents may be the same or different. Thus, in one embodiment, the phenyl
25 xanthene dyes comprise any fluorescein, rhodol, or rhodamine that comprises a C9 phenyl ring
26 comprising the following structure:



1
2 where at least one of R¹¹ or R¹⁵ is selected from alkyl, heteroalkyl, alkoxy, halo, haloalkyl,
3 amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl.

4 The remaining carbons on the C9 phenyl ring can, independently or one another, be
5 unsubstituted or substituted with any group having no more than 40 atoms and typically no more
6 than 25 atoms. Illustrative substituent groups that can be positioned at carbons C12, C13 and/or
7 C14 include alkyl, heteroalkyl, aryl, heteroaryl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano,
8 isocyano, cyanato, mercaptocyanato, nitro, sulfinyl, sulfonyl, sulfonamide, carboxyl, and
9 carboxyamide. Accordingly, in another embodiment, at least one of R¹¹ or R¹⁵ is substituted as
10 described above and the remainder of R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are, independently of one
11 another, selected from hydrogen, alkyl, heteroalkyl, aryl, heteroaryl, alkoxy, halo, haloalkyl,
12 amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, sulfinyl, sulfonyl,
13 sulfonamide, carboxyl, and carboxyamide.

14 It has been discovered that phenyl xanthene dyes including C9 phenyl that are substituted
15 with halo, haloalkyl, alkoxy and/or nitrile substituents exhibit especially good fluorescent
16 properties, particularly when placed at the C11 and/or C15 carbons. Accordingly, in another
17 embodiment, at least one of R¹¹ and R¹⁵ is selected from an alkoxy, halo, haloalkyl and/or
18 nitrile. In yet another embodiment, R¹¹ and R¹⁵ are each, independently of one another, an
19 alkoxy, halo, haloalkyl and/or nitrile.

20 In still another embodiment, at least one of R¹¹ and R¹⁵ is selected from an alkoxy, halo
21 and/or haloalkyl and the remainder of R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are, independently of one
22 another, selected from hydrogen, alkoxy, halo and/or haloalkyl. In another embodiment, R¹¹ and
23 R¹⁵ are each, independently of one another, an alkoxy, halo and/or haloalkyl and the remainder
24 of R¹², R¹³ and R¹⁴ are, independently of one another, selected from hydrogen, alkoxy, halo
25 and/or haloalkyl. Any alkoxy and/or halo and/or haloalkyl groups present on the lower phenyl
26 ring may be the same or different. However, in one embodiment, any alkoxy and/or halo and or
27 haloalkyl groups present on the lower phenyl ring is identical to any other alkoxy and/or halo

1 and/or haloalkyl groups present on the phenyl ring. Furthermore, in one embodiment, the lower
2 phenyl ring is only substituted with hydrogen, alkoxy, halo and/or haloalkyl groups.

3 Especially suitable alkoxy groups include (C1 to C20) oxyalkyls, particularly methoxy.
4 In one embodiment, the phenyl ring is only substituted with hydrogen and identical alkoxy
5 groups. In one embodiment at least two groups on the phenyl ring are alkoxy. In another
6 embodiment at least three groups on the phenyl ring are alkoxy. In another embodiment at least
7 four groups on the phenyl ring are alkoxy. In another embodiment all of the groups on the
8 phenyl ring are alkoxy.

9 Especially suitable halos include chloro and fluoro groups. In one embodiment, the
10 phenyl ring is only substituted with hydrogen and identical halo groups, such as fluoro or chloro.
11 In one embodiment at least two groups on the phenyl ring are halo. In another embodiment at
12 least three groups on the phenyl ring are halo. In another embodiment at least four groups on the
13 phenyl ring are halo. In another embodiment all of the groups on the phenyl ring are halo.

14 Especially suitable haloalkyls include -CF₃. Accordingly, in one embodiment, the
15 phenyl ring is only substituted with hydrogen and -CF₃ groups. In one embodiment at least two
16 groups on the phenyl ring are haloalkyl. In another embodiment at least three groups on the
17 phenyl ring are haloalkyl. In another embodiment at least four groups on the phenyl ring are
18 alkoxy. In another embodiment all of the groups on the phenyl ring are haloalkyl.

19 Embodiments where the C9 phenyl ring is substituted at both the C11 and C15 carbons
20 also exhibit especially good fluorescent properties. Accordingly, in one embodiment, R¹¹ and
21 R¹⁵ are each, independently of one another, selected from alkyl, heteroalkyl, alkoxy, halo,
22 haloalkyl, amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl. The
23 remaining carbons on the phenyl need not be substituted and, if substituted, the substituents
24 may, independently, be the same or different when compared to R¹¹ and/or R¹⁵.

25 Embodiments where the C9 phenyl ring is identically substituted at both carbons ortho to
26 the point of phenyl ring's attachment to the remainder of the phenyl xanthene dye also exhibit
27 desirable fluorescent properties. Accordingly, in another embodiment, R¹¹ and R¹⁵ are identical.
28 Once again, the remaining carbons on the phenyl need not be substituted and, if substituted, the
29 substituents may, independently, be the same or different when compared to R¹¹ and R¹⁵. In one
30 embodiment, any substituents on the lower phenyl ring are identical.

31 Symmetry appears to be an important factor in selecting optimal C9 phenyl rings. In this
32 regard, the symmetry is relative to an imaginary axis running from the lower phenyl ring's point
33 of attachment (*i.e.*, the 10-carbon) to the remainder of the phenyl xanthene dye (*i.e.*, the 9-
34 carbon) through a point *para* to the attachment (*i.e.*, the 13-carbon). Accordingly, in one

1 embodiment, R¹¹ and R¹⁵ are identical and the remainder of R¹², R¹³ and R¹⁴ are, identically,
2 either hydrogen or a substituent different from R¹¹ and R¹⁵. In another embodiment R¹¹, R¹³,
3 and R¹⁵ are identical and the remainder of R¹², R¹³, and R¹⁴ are, identically, either hydrogen or a
4 substituent different from R¹¹, R¹³ and R¹⁵. In yet another embodiment, R¹¹, R¹², R¹⁴ and R¹⁵ are
5 identical and R¹³ is either hydrogen or a substituent different from R¹¹, R¹², R¹⁴ and R¹⁵. In still
6 another embodiment, R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are all identical. Optimal lower phenyl rings
7 include those where the ring exhibits one of the aforementioned symmetries and R¹¹ and R¹⁵ are
8 selected from the same alkoxy and/or halo and/or haloalkyl groups.

9 The C9 phenyl ring departs from known C9 phenyl rings in phenyl xanthene dyes in
10 many ways. For example, as evident from the patent literature, it is conventional wisdom to
11 substitute the *ortho* phenyl position with a carboxyl or sulfonyl group, or some derivative
12 thereof, such as an ester, amide, acid halide or salt. See, e.g., U.S. Patent No. 6,248,884, U.S.
13 Patent No. 6,229,055, U.S. Patent No. 5,936,087, U.S. Patent No. 5,847,162, U.S. Patent No.
14 5,840,999, U.S. Patent No. 5,750,409, U.S. Patent No. 5,654,442, U.S. Patent No. 5,442,045,
15 U.S. Patent No. 5,410,053, U.S. Patent No. 5,366,860, U.S. Patent No. 5,231,191, U.S. Patent
16 No. 5,188,934, U.S. Patent No. 5,066,580, U.S. Patent No. 4,481,136 and U.S. Patent No.
17 4,439,356. This a result of conventional synthetic procedures. However, the instant C9 phenyl
18 rings do not contain the aforementioned *ortho* carboxyl, *ortho* sulfonyl, or an ester, amide, acid
19 halide, or salt thereof.

20 The C9 phenyl ring can be connected to any fluorescein, rhodol or rhodamine type upper
21 ring. Rhodamines are phenyl xanthenes that additionally comprise an exocyclic amine group
22 and an exocyclic imminium group. Rhodols are phenyl xanthenes that additionally comprise an
23 exocyclic amine group and an exocyclic oxo group. Fluoresceins are phenyl xanthenes that
24 additionally comprise an exocyclic hydroxyl group and an exocyclic oxo group. The phenyl
25 xanthene dyes described can employ any fluorescein, rhodol and rhodamine type upper ring as
26 long as the C9 phenyl attached thereto is as described herein. Accordingly, in one embodiment,
27 the phenyl xanthene dye comprises a fluorescein type upper ring. In another embodiment, the
28 phenyl xanthene dye comprises a rhodol type upper ring. In another embodiment, the phenyl
29 xanthene dye comprises a rhodamine type upper ring.

30 Suitable fluorescein, rhodamine and rhodol type upper rings are provided, for example,
31 in U.S. Patent No. 6,248,884, U.S. Patent No. 6,229,055, U.S. Patent No. 5,936,087, U.S. Patent
32 No. 5,847,162, U.S. Patent No. 5,840,999, U.S. Patent No. 5,750,409, U.S. Patent No.
33 5,654,442, U.S. Patent No. 5,442,045, U.S. Patent No. 5,410,053, U.S. Patent No. 5,366,860,
34 U.S. Patent No. 5,231,191, U.S. Patent No. 5,188,934, U.S. Patent No. 5,066,580, U.S. Patent

1 No. 4,481,136 and U.S. Patent No. 4,439,356, all of which relate to phenyl xanthenes and all of
2 which are hereby incorporated by reference. However, the upper ring is not limited to the
3 structures described in these patents. As stated, any fluorescein, rhodol or rhodamine type upper
4 ring can be employed as long as it is attached to the C9 phenyl ring described herein.

5 Furthermore, as known in the art, phenyl xanthene dyes can be extended to include a 3,4-
6 and/or a 5,6-benzo substituent (*see, e.g.*, U.S. Patent No. 6,248,884, U.S. Patent No. 5,750,409
7 and U.S. Patent No. 5,066,580). In "extended" fluoresceins, rhodols and rhodamines, the
8 exocyclic amine or hydroxyl group and/or the exocyclic imminium or oxo group are attached to
9 any present 3,4- and/or 5,6-benzo substituents. These "extended" fluorescein, rhodol and
10 rhodamine rings can also be employed in the invention as long as they comprise the C9 phenyl
11 described herein. Accordingly, the "fluorescein," "rhodol" and "rhodamine" as used herein
12 embrace extended structures.

13 In one embodiment, the phenyl xanthene dyes not only contain the new lower phenyl
14 ring but also contain sufficient lipophilic groups to make the phenyl xanthenes lipid soluble.
15 This is especially beneficial when the phenyl xanthenes are used, for example, to imbibe
16 hydrophobic polymeric particles that are useful in aqueous assays. Non-limiting examples of
17 such polymeric particles include crosslinked and uncrosslinked polystyrene particles and
18 styrene-(meth) acrylic acid copolymers. As evident to one of ordinary skill in the art, an
19 unlimited variety of particles for use in assays are commercially available, including particles
20 that are functionalized and/or paramagnetic and/or conjugated with a biological reagents. In
21 such embodiments, the degree of lipid solubility required for the phenyl xanthene dye
22 necessarily varies as a function of the polymer utilized, the aqueous solvent or solvent system
23 employed in the assay in which the polymeric particle is to be used, and the conditions (*e.g.*,
24 time, temperature, pressure, pH, etc.) under which the assay is run. Suitable degrees of lipid
25 solubility are easily determined by methods known in the art. For example, suitable lipid
26 solubility can be determined by a partition test wherein a known quantity of dye in organic
27 solvent is combined with the aqueous solvent or solvent system used in the assay. If a partition
28 results and, under the conditions used in the assay, there is no appreciable crossing by the dye
29 into the solvent or solvent system, then the dye is sufficiently lipid soluble. Put another way, the
30 lipid soluble phenyl xanthene dye should be sufficiently lipid soluble such that it is capable of
31 being imbibed into the polymer when dissolved in an organic solvent or solvent system and,
32 when the dyed polymer is subjected to the aqueous conditions of the assay, the dye should resist
33 leaching out of the polymer to any degree that significantly impacts the fluorescent signature of
34 the dye imbibed polymer or the results of the assay.

1 In those embodiments where the phenyl xanthene dyes are lipid soluble rhodamines, one
2 or both of the exocyclic amine and exocyclic imminium nitrogens are often substituted with one
3 or more lipophilic groups designed to impart to the rhodamine lipophilic characteristics or
4 properties. Thus, useful dyes include rhodamines that comprise the lower phenyl ring described
5 above and also comprise one or two lipophilic substituents at the exocyclic amine nitrogen
6 and/or one or two lipophilic substituents at the exocyclic imminium nitrogen. In one
7 embodiment, both the exocyclic amine nitrogen and the exocyclic imminium nitrogen are
8 substituted with a lipophilic group. In another embodiment, the exocyclic amine nitrogen and
9 the exocyclic imminium nitrogen are both substituted with two lipophilic groups. The lipophilic
10 groups, whether attached to the same or different exocyclic nitrogen, may be the same or
11 different. In one embodiment, the lipophilic groups on the exocyclic nitrogens are the same.

12 In those embodiments where the phenyl xanthene dyes are lipid soluble rhodols, the
13 exocyclic amine nitrogen is often substituted with one or more lipophilic groups designed to
14 impart to the rhodol lipophilic characteristics or properties. Thus, useful dyes include rhodols
15 that comprise the C9 phenyl described herein and also comprise one or two lipophilic
16 substituents at the exocyclic amine nitrogen. In one embodiment, the exocyclic amine nitrogen
17 is substituted with one lipophilic group. In another embodiment, the exocyclic amine nitrogen is
18 substituted with two lipophilic groups. If there are two lipophilic groups on the exocyclic amine
19 nitrogen, the lipophilic groups may be same or different. In one embodiment, there are two
20 lipophilic groups on the exocyclic amine nitrogen that are the same.

21 Lipid-soluble phenyl xanthene dyes may include lipophilic substituents at other positions
22 as well. It is the net effect of the lipophilic substituents that determines whether the phenyl
23 xanthene dye is lipid soluble. This is especially true for fluoresceins which have no exocyclic
24 amine or imminium nitrogens.

25 Lipophilic substituents are groups that impart the resultant phenyl xanthene dye with
26 lipophilic characteristics or properties as denoted above. The nature of each lipophilic
27 substituent is not critical, as long as the resultant phenyl xanthene dye is lipid soluble. Non-
28 limiting examples of suitable lipophilic substituents include unsubstituted (C4-C20) alkyls, (C5-
29 C40) aryls, and (C6-C40) arylalkyls. Depending on the number of methylene and methine units
30 in the lipophilic substituent, the lipophilic substituent may also include pendant or internal polar
31 or hydrophilic groups. For example, a lipophilic substituent may include one or more internal
32 heteroatoms, such as one or more internal O, S, N or NH groups. As another example, a
33 lipophilic substituent may include one or more pendant polar or hydrophilic substituents, such as
34 one or more pendant halogen, -OH, -SH, -NH₂, -C(O)OH, -C(O)NH₂ or other polar or

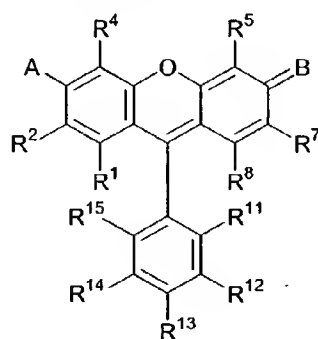
1 hydrophilic groups. Thus, lipophilic substituents may also include substituted (C4-C20) alkyl,
2 substituted (C5-C40) aryls and substituted (C6-C40) arylalkyls, as well as substituted and
3 unsubstituted (C4-C20) heteroalkyl, substituted and unsubstituted (C5-C40) heteroaryls and
4 substituted and unsubstituted (C6-C40) arylalkyls. The number of internal or pendant polar or
5 hydrophilic groups that may be included in a lipophilic substituent will depend upon, among
6 other factors, the number of methylene or methine groups included in the lipophilic substituent
7 and the number of lipophilic substituents on the phenyl xanthene dye. The nature and number of
8 lipophilic groups necessary to make a phenyl xanthene lipid soluble can vary from molecule to
9 molecule, and will be apparent to those of skill in the art.

10 Oftentimes, it is desirable to attach fluorescent dyes such as the phenyl xanthene dyes
11 described herein to substances such as solid supports, particles, and biological and non-
12 biological molecules (e.g., drugs, amino acids, peptides, polypeptides, proteins, nucleosides,
13 nucleotides, oligonucleotides, polynucleotides, carbohydrates, etc.) Thus, in one embodiment,
14 the various phenyl xanthene dyes described herein include one or more moieties suitable for
15 such attachment. Such moieties are expressed by the formula -S-LG where S is a direct bond or
16 a spacing moiety and LG is a linking group capable of forming a linkage with the substance to
17 be conjugated. The linking group LG may be any moiety capable of forming the linkage, which
18 may be covalent or non-covalent. For example, the linking group may be one member of a pair
19 of specific binding molecules that non-covalently bind one another, such as biotin and
20 avidin/streptavidin. Thus, in one embodiment, the linking group is biotin. Alternatively, the
21 linking group may be a functional group capable of forming a covalent linkage with a
22 "complementary" functional group, such as an electrophilic (or nucleophilic) group which is
23 capable of forming a covalent linkage with a complementary nucleophilic (or electrophilic)
24 group, although other groups may be used depending on the desired linking chemistry, as is well
25 known in the art. The linking group may be attached directly to the phenyl xanthene dye or it
26 may be spaced away from the phenyl xanthene dye by way of spacing moiety "S." As will be
27 appreciated by skilled artisans, the nature and composition of the spacing moiety is not critical
28 and may depend upon the particular application. The linking group, whether attached directly or
29 spaced away *via* spacing moiety "S," may be attached to any available position of the phenyl
30 xanthene dye. For example, the linking group may be attached to any available position on the
31 upper ring or the lower ring. In one embodiment, the linking group -S-LG is attached to the C2,
32 C4, C5, or C7 position of the upper ring. In another embodiment, the linking group -S-LG is
33 attached to the C12, C13 or C14 position of the lower ring.

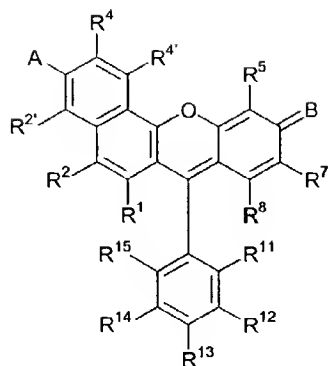
Alternatively, the lipid-soluble phenyl xanthene dyes may be linked to a conjugated substance. In this embodiment, at least one substituent on the phenyl xanthene dye is $-S^1-LK-S^2-CS$, where S^1 and S^2 are, independently of one another, a direct bond or a spacing moiety, LK represents a linkage, which may be a bond or another type of linkage, and CS is a conjugated substance. Non-limiting examples of substances that can be conjugated include glass substrates, metal substrates, polymeric substrates, biomolecules, haptens, drugs, poisons, vitamins, antigens, and pathogens. Once again, the linker will vary depending the identity of the conjugated substance.

Similarly, the phenyl xanthene dye may be part of an energy transfer ("ET") network comprising, for example, from two to four dyes covalently attached to one another that transfer energy to generate a longer Stoke's shift. In other words, the phenyl xanthene dye may be part of series of dyes that are covalently attached to one another. One example of an ET network would be a fluorescence resonance energy transfer ("FRET") dye. In this embodiment, at least one substituent on the phenyl xanthene dye is $-S^1-LK-S^2-D$, where S^1 and S^2 are, independently of one another, a direct bond or a spacing moiety, LK represents a linkage, which may be a bond or another type of linkage, and D is a dye. Linkages for covalently attaching phenyl xanthene dyes to other dyes are known in the art, as are suitable locations for attachment to the phenyl xanthene dyes (*see, e.g.*, U.S. Patent Nos. 5,800,996 and 5,863,727). In one embodiment, each dye in the energy transfer network is within 5 to 100 Å of the neighboring dye or dyes in the network to which it is covalently attached. In such embodiments, the phenyl xanthene dye can be the donor, acceptor, or an intermediate dye in the network.

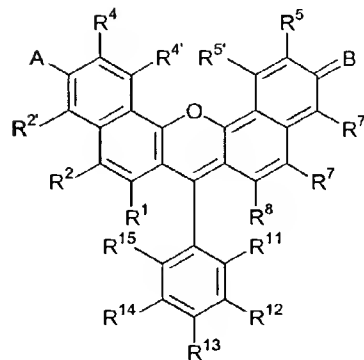
In a more particular embodiment, the phenyl xanthene dyes are any fluorescent dye that comprises one of the following "core structures:"



(I)



(II)



(III)

1 where A is $-\text{OH}$ or NR^3R^3 , where B is a $=\text{O}$ or $=\text{N}^{\oplus}\text{R}^6\text{R}^6$, where R^{11} and R^{15} are,
2 independently of one another, selected from alkyl, heteroalkyl, alkoxy, halo, haloalkyl, amino,
3 alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl, and the remainder of
4 $\text{R}^1, \text{R}^2, \text{R}^2, \text{R}^3, \text{R}^3, \text{R}^4, \text{R}^4, \text{R}^5, \text{R}^5, \text{R}^6, \text{R}^6, \text{R}^7, \text{R}^7, \text{R}^8, \text{R}^{12}, \text{R}^{13}$, and R^{14} are, independently of
5 one another, selected from hydrogen and a substituent having no more than 40 atoms, and
6 typically no more than 25 atoms. In one embodiment, the phenyl xanthene dye is lipid soluble.
7 In another embodiment, one or more of the remainder of $\text{R}^1, \text{R}^2, \text{R}^2, \text{R}^3, \text{R}^3, \text{R}^4, \text{R}^4, \text{R}^5, \text{R}^5,$
8 $\text{R}^6, \text{R}^6, \text{R}^7, \text{R}^7, \text{R}^8, \text{R}^{12}, \text{R}^{13}$, and R^{14} may be $-\text{S}-\text{LG}$ where S is a direct bond or a spacing
9 moiety and LG is a linking group. In another embodiment one or more of the remainder of $\text{R}^1,$
10 $\text{R}^2, \text{R}^2, \text{R}^3, \text{R}^3, \text{R}^4, \text{R}^4, \text{R}^5, \text{R}^5, \text{R}^6, \text{R}^6, \text{R}^7, \text{R}^7, \text{R}^8, \text{R}^{12}, \text{R}^{13}$, and R^{14} may be $-\text{S}^1-\text{LK}-\text{S}^2-\text{CS}$,
11 where S^1 and S^2 are each, independently of one another, a direct bond or a spacing moiety, LK is
12 a linkage, and CS is a conjugated substance.

13 The phenyl xanthene dyes are useful in any commonly known application for dyes. For
14 example the dyes are useful as fluorescent labels for automated DNA sequencing,
15 oligonucleotide hybridization methods, detection of polymerase-chain reaction products,
16 immunoassays, and the like. In addition, the dyes may be imbibed into polymeric particles for
17 use in the standardization of fluorescence-based instrumentation, as a biological tracer, and in
18 the detection and analysis of biomolecules. In these latter applications, it is often desirable for
19 the dyes to be lipid soluble as previously discussed.

20 Other aspects of the invention will be apparent to those of ordinary skill in the art in view
21 of the disclosure provided herein.

22 4. BRIEF DESCRIPTION OF THE DRAWINGS

23 FIG. 1 illustrates the synthesis of phenyl xanthene dyes;

24 FIGS. 2A and 2B illustrate the synthesis of extended phenyl xanthene dyes;

1 **FIG. 3** illustrates the synthesis of fluoresceins;

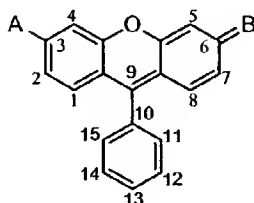
2 **FIG. 4** illustrates the synthesis of extended fluoresceins; and

3 **FIG. 5** illustrates the synthesis of a rhodol.

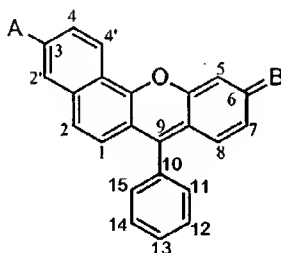
4 **5. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

5 **5.1 Numbering System**

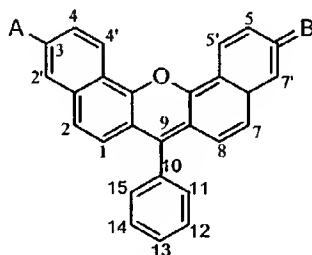
6 For the purposes of the present application, carbon atoms in phenyl xanthenes such as
7 fluoresceins, rhodols and rhodamines, or extended versions thereof, are numbered in the
8 following manner:



9
10 (Formula 1)



11
12 (Formula 2)



13
14 (Formula 3)

15 where A is either a hydroxyl (-OH) or an amine (-NH₂) and B is either an oxo (=O) or an
16 iminium (=NH₂⁺).

17 **5.2 Definitions**

18 As used herein, the following terms are intended to have the following meanings:

19 “Phenyl Xanthene Dye,” as used herein, refers to any dye that comprises a xanthene ring
20 or an extended xanthene ring that is substituted with a C9 phenyl group, an exocyclic amine or

1 hydroxyl group and an exocyclic imminium or oxo group, as shown in formulae (1), (2) and (3)
2 above. As known in the art, various substitutions may be made for the hydrogens on any of the
3 1-, 2-, 2'-, 4-, 4'-, 5'-, 5-, 7'-, 7-, 8-, 11-, 12-, 13-, 14-, and 15-carbons, as well as any hydrogens
4 on any exocyclic amine or exocyclic imminium present. Substitutions can be independently
5 selected from any of a wide variety of the same or different groups known in the art including,
6 but not limited to, -X, -R^S, -OR^S, -SR^S, -NR^SR^S, perhalo, (C1-20) alkyl, -CX₃, -CN, -OCN, -
7 SCN, -NCO, -NCS, -NO, --NO₂, --N₃, --S(O)₂O⁻, -S(O)₂OH, --S(O)₂R^S, -C(O)R^S, -C(O)X, -
8 C(S)R^S, -C(S)X, -C(O)OR^S, -C(S)OR^S, -C(O)SR^S, -C(S)SR^S, -C(O)NR^SR^S, -C(S)NR^SR^S and -
9 C(NR^S)NR^SR^S, where each X is independently a halogen (e.g., fluoride or chloride), and each R^S
10 is independently hydrogen, (C1-C20) alkyl or heteroalkyl, (C5-C20) aryl or heteroaryl, and (C6-
11 C40) arylalkyl or heteroarylalkyl. Any of the aforementioned substituents can, in turn, be
12 further substituted with one or more of the same or different substituents.

13 Moreover, the 1- and 2- substituents, or the 2- and 2'- substituents, and/or the 7' and 7
14 substituents or the 7- and 8- substituents, can be taken together to form substituted or
15 unsubstituted (C5-C20) benzo, naphtho or polycyclic aryleno bridges. The bridges may, in turn,
16 be further substituted, for example, with any of the substituents R^S above.

17 When A is an amine and/or B is an imminium, the exocyclic nitrogen or nitrogens can be
18 included in 5 or 6 membered rings involving the nitrogen atom and an adjacent carbon atom on
19 the xanthene dye. The rings may, in turn, be further substituted, for example, with any of the
20 substituents R^S above.

21 "Rhodamine," as used herein, is a specific type of phenyl xanthene dye. Rhodamines
22 embrace any substituted or unsubstituted dye that comprises one of formulae (1), (2) and (3)
23 above, where A is a substituted or unsubstituted amine group and B is a substituted or
24 unsubstituted imminium group. Examples of the various substitutions that may be made for
25 hydrogens at the 1-, 2-, 2'-, 4-, 4'-, 5'-, 5-, 7'-, 7-, and 8- carbons, as well as hydrogens at the
26 amine and imminium nitrogens are illustrated, for example, in U.S. Patent No. 6,372,907, U.S.
27 Patent No. 6,248,884, U.S. Patent No. 5,936,087, U.S. Pat. No. 5,847,162, U.S. Pat. No.
28 5,840,999, U.S. Patent No. 5,750,409, U.S. Patent No. 5,410,053, U.S. Pat. No. 5,366,860, and
29 U.S. Pat. No. 5,231,191.

30 "Rhodol," as used herein, is another specific type of phenyl xanthene dye. Rhodamines
31 embrace any substituted or unsubstituted dye that comprises one of formulae (1), (2) and (3)
32 above, where A is a substituted or unsubstituted amine group and B is an oxo group. Examples
33 of the various substitutions that may be made for hydrogens at the 1-, 2-, 2'-, 4-, 4'-, 5'-, 5-, 7'-,
34 7-, and 8- carbons, as well as hydrogens at the amine nitrogen are illustrated, for example, in

1 U.S. Patent No. 6,372,907, U.S. Patent U.S. Patent No. 6,229,055, U.S. Patent No. 6,008,379,
2 U.S. Patent No. 5,840,999, and U.S. Patent No. 5,442,045.

3 "Fluorescein," as used herein, is another specific type of phenyl xanthene dye.
4 Rhodamines embrace any substituted or unsubstituted dye that comprises one of formulae (1),
5 (2) and (3) above, where A is a hydroxyl group and B is an oxo group. Examples of the various
6 substitutions that may be made for hydrogens at the 1-, 2-, 2'-, 4-, 4'-, 5'-, 5-, 7'-, 7-, and 8-
7 carbons are illustrated, for example, in U.S. Patent No. 6,229,055, U.S. Pat. No. 5,840,999, U.S.
8 Pat. No. 5,654,442, U.S. Patent No. 5,750,409, U.S. Pat. No. 5,188,934, U.S. Patent No.
9 5,066,580, U.S. Pat. No. 4,481,136, and U.S. Pat. No. 4,439,356.

10 "Fluorescent Dye" or "Fluorescer" or "Fluorochrome" or "Fluorophore" as used
11 interchangeably herein refer to molecules that absorb electromagnetic radiation at one
12 wavelength and emit electromagnetic radiation at another wavelength in passing from a higher
13 to a lower electronic state.

14 "Carboxyl" as used herein, is defined to include not only the carboxyl group (-COOH or
15 -CO₂H) but also carboxylate radicals (-CO₂⁻).

16 "Sulfonyl," as used herein, is defined to include not only the sulfonyl group (-SO₂OH or
17 -SO₃H), but also sulfonate radicals (-SO₃⁻).

18 "Biomolecule" as used herein refers to a molecule of a type typically found in a
19 biological system, whether such molecule is naturally occurring or the result of some external
20 disturbance of the system (e.g., a disease, poisoning, genetic manipulation, etc.), as well as
21 synthetic analogs and derivatives thereof. Non-limiting examples of biomolecules include
22 amino acids (naturally occurring or synthetic), peptides, polypeptides, glycosylated and
23 unglycosylated proteins (e.g., polyclonal and monoclonal antibodies, receptors, interferons,
24 enzymes, etc.), nucleosides, nucleotides, oligonucleotides (e.g., DNA, RNA, PNA oligos),
25 polynucleotides (e.g., DNA, cDNA, RNA, etc.), carbohydrates, hormones, haptens, steroids,
26 toxins, etc. Biomolecules may be isolated from natural sources, or they may be synthetic.

27 "Alkyl" by itself or as part of another substituent refers to a saturated or unsaturated
28 branched, straight-chain or cyclic monovalent hydrocarbon radical having the stated number of
29 carbon atoms (i.e., C1-C6 means one to six carbon atoms) that is derived by the removal of one
30 hydrogen atom from a single carbon atom of a parent alkane, alkene or alkyne. Typical alkyl
31 groups include, but are not limited to, methyl; ethyls such as ethanyl, ethenyl, ethynyl; propyls
32 such as propan-1-yl, propan-2-yl, cyclopropan-1-yl, prop-1-en-1-yl, prop-1-en-2-yl,
33 prop-2-en-1-yl, cycloprop-1-en-1-yl; cycloprop-2-en-1-yl, prop-1-yn-1-yl, prop-2-yn-1-yl, etc.;
34 butyls such as butan-1-yl, butan-2-yl, 2-methyl-propan-1-yl, 2-methyl-propan-2-yl,

1 cyclobutan-1-yl, but-1-en-1-yl, but-1-en-2-yl, 2-methyl-prop-1-en-1-yl, but-2-en-1-yl ,
2 but-2-en-2-yl, buta-1,3-dien-1-yl, buta-1,3-dien-2-yl, cyclobut-1-en-1-yl, cyclobut-1-en-3-yl,
3 cyclobuta-1,3-dien-1-yl, but-1-yn-1-yl, but-1-yn-3-yl, but-3-yn-1-yl, etc.; and the like. Where
4 specific levels of saturation are intended, the nomenclature "alkanyl," "alkenyl" and/or
5 "alkynyl" is used, as defined below. In preferred embodiments, the alkyl groups are (C1-C20)
6 alkyl.

7 "Heteroalkyl," by itself or as part of another substituent refers to an alkyl in which one or
8 more of the carbon atoms are each independently replaced with the same or different
9 heteroatoms or heteroatomic groups. Typical heteroatoms and/or heteroatomic groups which
10 can replace the carbon atoms include, but are not limited to, -O-, -S-, -S-O-, -NR^m-, -PH-,
11 -S(O)-, -S(O)₂-, -S(O) NR^m-, -S(O)₂NR^m-, and the like, including combinations thereof, where
12 each R^m is independently hydrogen or (C1-C6) alkyl.

13 "Aryl" by itself or as part of another substituent refers to a monovalent aromatic
14 hydrocarbon group having the stated number of carbon atoms (*i.e.*, C5-C15 means from 5 to 15
15 carbon atoms) derived by the removal of one hydrogen atom from a single carbon atom of a
16 parent aromatic ring system. Typical aryl groups include, but are not limited to, groups derived
17 from aceanthrylene, acenaphthylene, acephenanthrylene, anthracene, azulene, benzene,
18 chrysene, coronene, fluoranthene, fluorene, hexacene, hexaphene, hexalene, *as*-indacene,
19 *s*-indacene, indane, indene, naphthalene, octacene, octaphene, octalene, ovalene,
20 penta-2,4-diene, pentacene, pentalene, pentaphene, perylene, phenalene, phenanthrene, picene,
21 pleiadene, pyrene, pyranthrene, rubicene, triphenylene, trinaphthalene, and the like, as well as
22 the various hydro isomers thereof. In preferred embodiments, the aryl group is (C5-C15) aryl,
23 with (C5-C10) being even more preferred. Particularly preferred aryls are phenyl and naphthyl.

24 "Arylaryl" by itself or as part of another substituent refers to a monovalent hydrocarbon
25 group derived by the removal of one hydrogen atom from a single carbon atom of a ring system
26 in which two or more identical or non-identical parent aromatic ring systems are joined directly
27 together by a single bond, where the number of such direct ring junctions is one less than the
28 number of parent aromatic ring systems involved. Typical arylaryl groups include, but are not
29 limited to, biphenyl, triphenyl, phenyl-naphthyl, binaphthyl, biphenyl-naphthyl, and the like.
30 Where the number of carbon atoms in an arylaryl group are specified, the numbers refer to the
31 carbon atoms comprising each parent aromatic ring. For example, (C5-C15) arylaryl is an
32 arylaryl group in which each aromatic ring comprises from 5 to 15 carbons, *e.g.*, biphenyl,
33 triphenyl, binaphthyl, phenylnaphthyl, etc. Preferably, each parent aromatic ring system of an
34 arylaryl group is independently a (C5-C15) aromatic, more preferably a (C5-C10) aromatic.

1 Also preferred are arylaryl groups in which all of the parent aromatic ring systems are identical,
2 *e.g.*, biphenyl, triphenyl, binaphthyl, trinaphthyl, etc.

3 "Arylalkyl" by itself or as part of another substituent refers to an acyclic alkyl group in
4 which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or *sp*³ carbon
5 atom, is replaced with an aryl group. Typical arylalkyl groups include, but are not limited to,
6 benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl,
7 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. Where specific
8 alkyl moieties are intended, the nomenclature arylalkanyl, arylakenyl and/or arylalkynyl is used.
9 In preferred embodiments, the arylalkyl group is (C6-C21) arylalkyl, *e.g.*, the alkanyl, alkenyl or
10 alkynyl moiety of the arylalkyl group is (C1-C6) and the aryl moiety is (C5-C15). In
11 particularly preferred embodiments the arylalkyl group is (C6-C13), *e.g.*, the alkanyl, alkenyl or
12 alkynyl moiety of the arylalkyl group is (C1-C3) and the aryl moiety is (C5-C10).

13 "Heteroaryl" by itself or as part of another substituent refers to a monovalent
14 heteroaromatic group having the stated number of ring atoms (*e.g.*, "5-14 membered" means
15 from 5 to 14 ring atoms) derived by the removal of one hydrogen atom from a single atom of a
16 parent heteroaromatic ring system. Typical heteroaryl groups include, but are not limited to,
17 groups derived from acridine, benzimidazole, benzisoxazole, benzodioxan, benzodioxole,
18 benzofuran, benzopyrone, benzothiadiazole, benzothiazole, benzotriazole, benzoxazine,
19 benzoxazole, benzoxazoline, carbazole, β -carboline, chromane, chromene, cinnoline, furan,
20 imidazole, indazole, indole, indoline, indolizine, isobenzofuran, isochromene, isoindole,
21 isoindoline, isoquinoline, isothiazole, isoxazole, naphthyridine, oxadiazole, oxazole, perimidine,
22 phenanthridine, phenanthroline, phenazine, phthalazine, pteridine, purine, pyran, pyrazine,
23 pyrazole, pyridazine, pyridine, pyrimidine, pyrrole, pyrrolizine, quinazoline, quinoline,
24 quinolizine, quinoxaline, tetrazole, thiadiazole, thiazole, thiophene, triazole, xanthene, and the
25 like, as well as the various hydro isomers thereof. In preferred embodiments, the heteroaryl
26 group is a 5-14 membered heteroaryl, with 5-10 membered heteroaryl being particularly
27 preferred.

28 "Heteroaryl-Heteroaryl" by itself or as part of another substituent refers to a monovalent
29 heteroaromatic group derived by the removal of one hydrogen atom from a single atom of a ring
30 system in which two or more identical or non-identical parent heteroaromatic ring systems are
31 joined directly together by a single bond, where the number of such direct ring junctions is one
32 less than the number of parent heteroaromatic ring systems involved. Typical
33 heteroaryl-heteroaryl groups include, but are not limited to, bipyridyl, tripyridyl, pyridylpurinyl,

bipurinyl, etc. Where the number of atoms are specified, the numbers refer to the number of atoms comprising each parent heteroaromatic ring systems. For example, 5-15 membered heteroaryl-heteroaryl is a heteroaryl-heteroaryl group in which each parent heteroaromatic ring system comprises from 5 to 15 atoms, *e.g.*, bipyridyl, tripyridyl, etc. Preferably, each parent heteroaromatic ring system is independently a 5-15 membered heteroaromatic, more preferably a 5-10 membered heteroaromatic. Also preferred are heteroaryl-heteroaryl groups in which all of the parent heteroaromatic ring systems are identical.

“Heteroarylalkyl” by itself or as part of another substituent refers to an acyclic alkyl group in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or *sp*³ carbon atom, is replaced with a heteroaryl group. Where specific alkyl moieties are intended, the nomenclature heteroarylalkanyl, heteroarylakenyl and/or heteroarylalkynyl is used. In preferred embodiments, the heteroarylalkyl group is a 6-21 membered heteroarylalkyl, *e.g.*, the alkanyl, alkenyl or alkynyl moiety of the heteroarylalkyl is (C1-C6) alkyl and the heteroaryl moiety is a 5-15-membered heteroaryl. In particularly preferred embodiments, the heteroarylalkyl is a 6-13 membered heteroarylalkyl, *e.g.*, the alkanyl, alkenyl or alkynyl moiety is (C1-C3) alkyl and the heteroaryl moiety is a 5-10 membered heteroaryl.

“Halogen” or “Halo” by themselves or as part of another substituent, unless otherwise stated, refer to fluoro, chloro, bromo and iodo.

“Haloalkyl” by itself or as part of another substituent refers to an alkyl group in which one or more of the hydrogen atoms is replaced with a halogen. Thus, the term “haloalkyl” is meant to include monohaloalkyls, dihaloalkyls, trihaloalkyls, etc. up to perhaloalkyls. For example, the expression “(C1-C2) haloalkyl” includes 1-fluoromethyl, difluoromethyl, trifluoromethyl, 1-fluoroethyl, 1,1-difluoroethyl, 1,2-difluoroethyl, 1,1,1-trifluoroethyl, perfluoroethyl, etc.

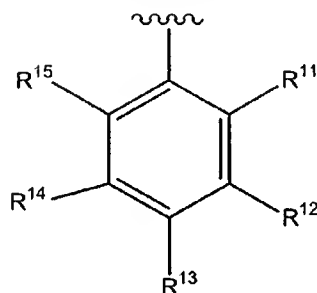
The above-defined groups may include prefixes and/or suffixes that are commonly used in the art to create additional well-recognized substituent groups. As examples, “alkyloxy” or “alkoxy” refers to a group of the formula -ORⁿ, “alkylamine” refers to a group of the formula -NHRⁿ and “dialkylamine” refers to a group of the formula -NRⁿRⁿ, where each Rⁿ is independently an alkyl. As another example, “haloalkoxy” or “haloalkyloxy” refers to a group of the formula -OR^p, where R^p is a haloalkyl.

5.3 Phenyl Xanthene Dyes

The phenyl xanthene dyes include any fluorescein, rhodol or rhodamine that comprises the C9 phenyl ring described herein. Accordingly, any rhodamine, rhodol and fluorescein type upper ring may be employed. Suitable rhodamine type upper rings are described in U.S. Patent

No. 6,248,884, U.S. Patent No. 6,229,055, U.S. Patent No. 5,936,087, U.S. Patent No. 5,847,162, U.S. Patent No. 5,840,999, U.S. Patent No. 5,750,409, U.S. Patent No. 5,654,442, U.S. Patent No. 5,442,045, U.S. Patent No. 5,410,053, U.S. Patent No. 5,366,860, U.S. Patent No. 5,231,191, U.S. Patent No. 5,188,934, U.S. Patent No. 5,066,580, U.S. Patent No. 4,481,136 and U.S. Patent No. 4,439,356, all of which are incorporated herein by reference. However, the upper rings are not limited by these patents. As stated, any fluorescein, rhodol or rhodamine type upper ring can be employed.

The C9 phenyl ring is substituted at one or both of carbons C11 or C15 with a group selected from alkyl, heteroalkyl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl. When both the C-11 and C-15 carbons are substituted, the substituents may be the same or different. Thus, in one embodiment, the phenyl xanthene dyes include any fluorescein, rhodol, or rhodamine upper ring that is substituted at the C9 carbon with a phenyl ring comprising the following structure:

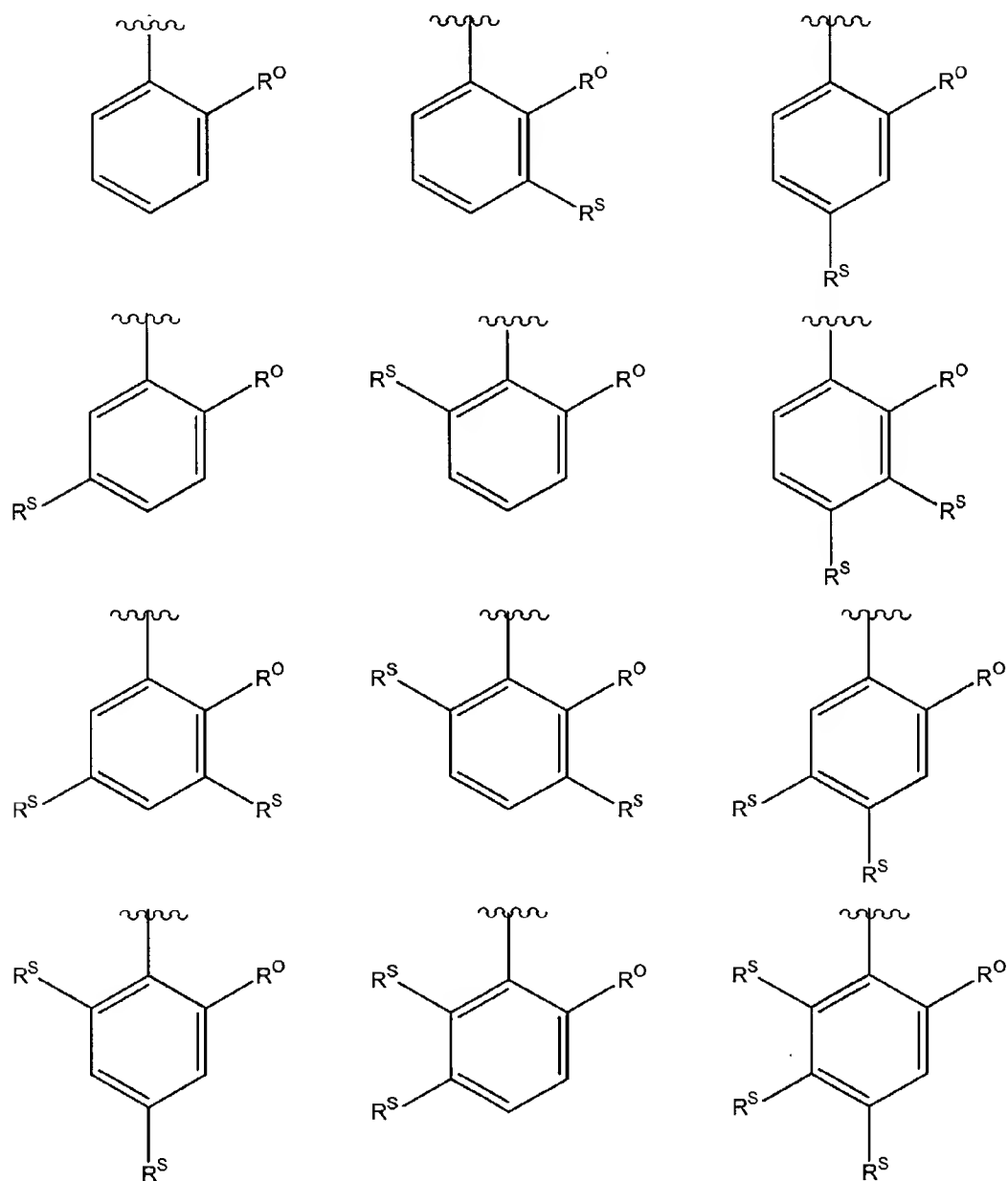


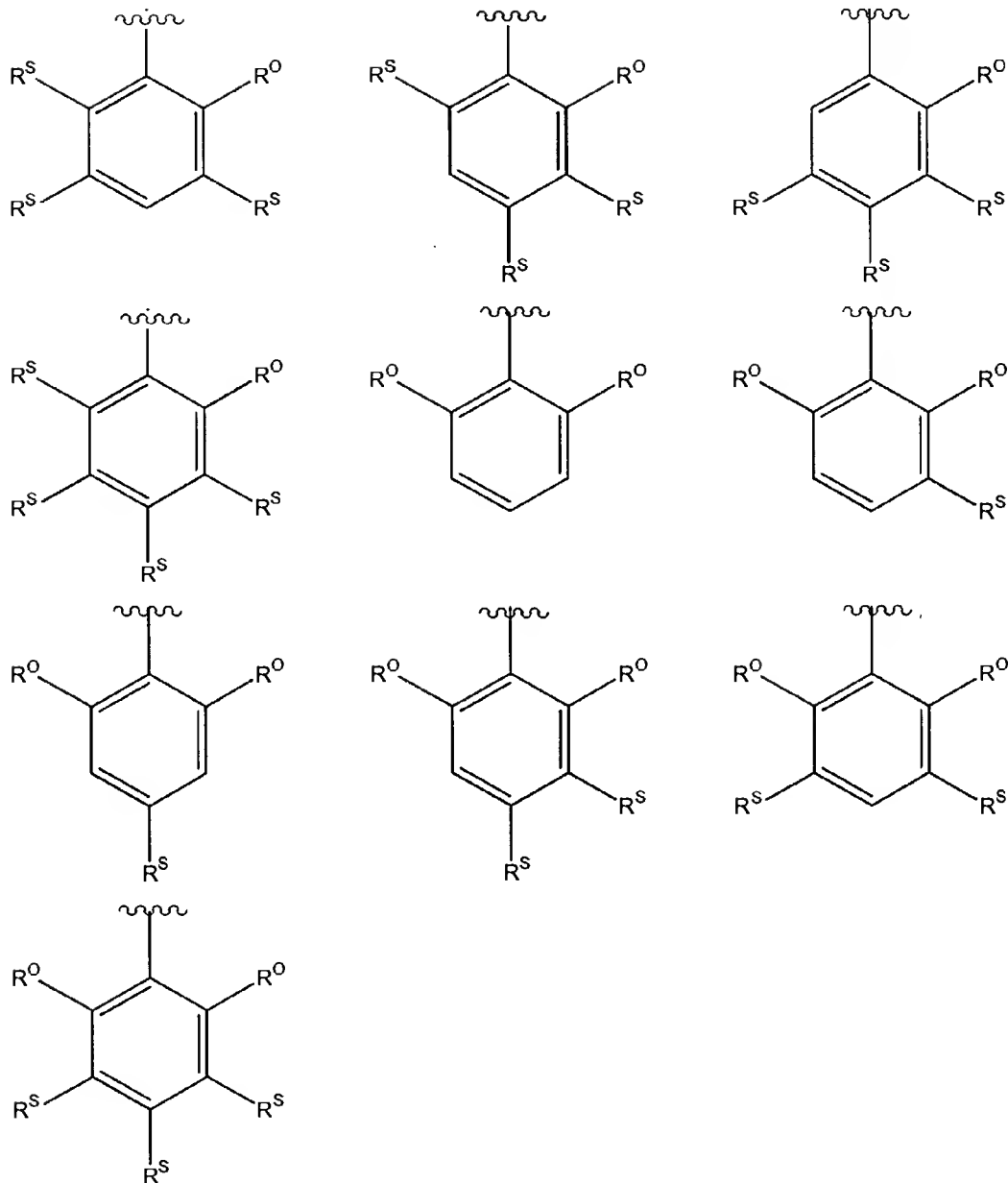
where at least one of R¹¹ or R¹⁵ is selected from alkyl, heteroalkyl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl.

The remaining carbons on the C9 phenyl ring can, independently or one another, be unsubstituted or substituted with any group having no more than 40 atoms and typically no more than 25 atoms. Illustrative substituent groups that can be positioned at carbons C12, C13 and/or C14 include alkyl, heteroalkyl, aryl, and heteroaryl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, sulfinyl, sulfonyl, sulfonamide, carboxyl and carboxamide. Accordingly, in another embodiment, at least one of R¹¹ or R¹⁵ is substituted as described above and the remainder of R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are, independently of one another, selected from hydrogen, alkyl, heteroalkyl, aryl, heteroaryl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, sulfinyl, sulfonyl, sulfonamide, carboxyl and carboxamide.

As long as at least one of R¹¹ and R¹⁵ is substituted as described above, substituents at the remaining carbons in the C9 phenyl ring may be absent or present in any conceivable

1 combination. This is illustrated in by the following exemplary C9 phenyl structures, wherein
2 one or both of R^{11} and R^{15} is R^O and the remaining carbons on the phenyl are either unsubstituted
3 or substituted with R^S , wherein each R^O , independently, is a group selected from alkyl,
4 heteroalkyl, alkoxy, halo, haloalkyl, amino, alkylthio, cyano, isocyano, cyanato,
5 mercaptocyanato, nitro, and sulfinyl, and each R^S , independently, is any substituent having up to
6 40 atoms.





1 It has been discovered that xanthene dyes including C9 phenyl that are substituted with
 2 halo, haloalkyl, alkoxy and/or nitrile substituents exhibit especially good fluorescent properties,
 3 particularly when placed at the C11 and/or C15 carbons. Accordingly, in another embodiment,
 4 at least one of R^{11} and R^{15} is selected from an alkoxy, halo, haloalkyl and/or nitrile. In yet
 5 another embodiment, R^{11} and R^{15} are each, independently of one another, an alkoxy, halo,
 6 haloalkyl and/or nitrile.

1 In still another embodiment, at least one of R¹¹ and R¹⁵ is selected from an alkoxy, halo
2 and/or haloalkyl and the remainder of R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are, independently of one
3 another, selected from hydrogen, alkoxy, halo and/or haloalkyl. In another embodiment, R¹¹ and
4 R¹⁵ are each, independently of one another, an alkoxy, halo and/or haloalkyl and the remainder
5 of R¹², R¹³ and R¹⁴ are, independently of one another, selected from hydrogen, alkoxy, halo
6 and/or haloalkyl. Any alkoxy and/or halo and/or haloalkyl group present on the lower phenyl
7 ring may be the same or different. However, in one embodiment, any alkoxy and/or halo and/or
8 halo alkyl group present on the lower phenyl ring is identical to any other alkoxy and/or halo
9 and/or haloalkyl group present on the phenyl ring. Furthermore, in one embodiment, the lower
10 phenyl ring is only substituted with hydrogen, alkoxy, halo and/or haloalkyl groups.

11 Especially suitable alkoxy groups include (C1 to C20) oxyalkyls, particularly methoxy.
12 In one embodiment, the phenyl ring is only substituted with hydrogen and identical alkoxy
13 groups. In one embodiment at least two groups on the phenyl ring are alkoxy. In another
14 embodiment at least three groups on the phenyl ring are alkoxy. In another embodiment at least
15 four groups on the phenyl ring are alkoxy. In a another embodiment all of the groups on the
16 phenyl ring are alkoxy.

17 Especially suitable halos include chloro and fluoro groups. In one embodiment, the
18 phenyl ring is only substituted with hydrogen and identical halo groups, such as fluoro or chloro.
19 In one embodiment at least two groups on the phenyl ring are halo. In another embodiment at
20 least three groups on the phenyl ring are halo. In another embodiment at least four groups on the
21 phenyl ring are halo. In a another embodiment all of the groups on the phenyl ring are halo.

22 Especially suitable haloalkyls include -CF₃. Accordingly, in one embodiment, the
23 phenyl ring is only substituted with hydrogen and -CF₃ groups. In one embodiment at least two
24 groups on the phenyl ring are haloalkyl. In another embodiment at least three groups on the
25 phenyl ring are haloalkyl. In another embodiment at least four groups on the phenyl ring are
26 alkoxy. In a another embodiment all of the groups on the phenyl ring are haloalkyl.

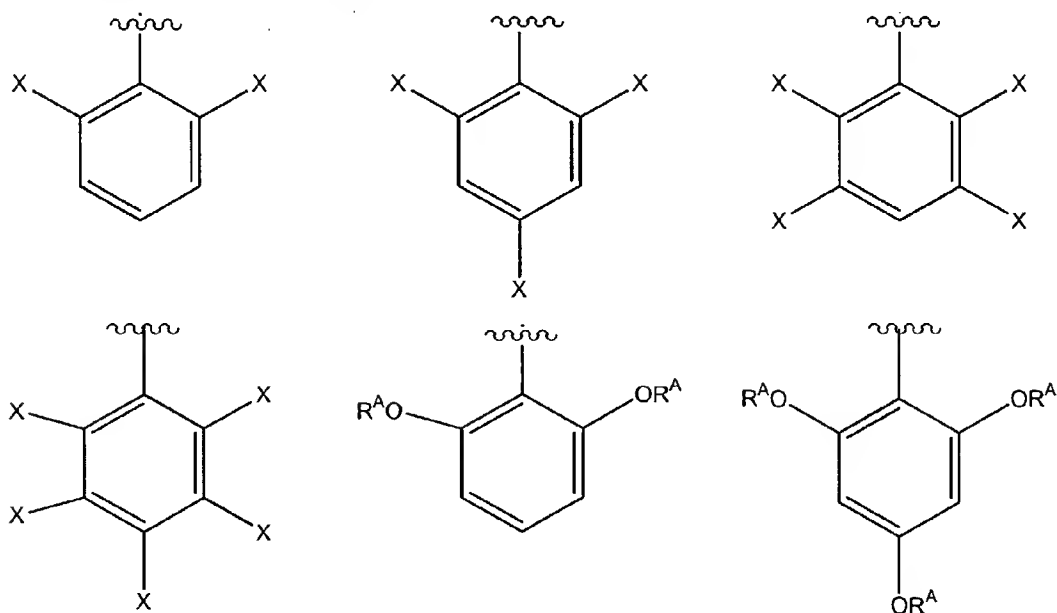
27 Embodiments where the C9 phenyl ring is substituted at both the C11 and C15 carbons
28 also exhibit especially good fluorescent properties. Accordingly, in one embodiment, R¹¹ and
29 R¹⁵ are each, independently of one another, selected from alkyl, heteroalkyl, alkoxy, halo,
30 haloalkyl, amino, alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl.
31 The remaining carbons on the phenyl need not be substituted and, if substituted, the substituents
32 may, independently, be the same or different when compared to R¹¹ and/or R¹⁵.

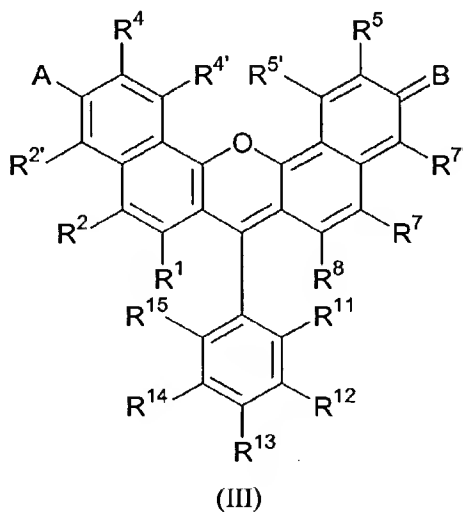
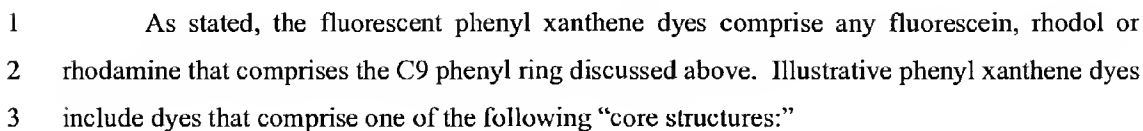
33 Embodiments where the C9 phenyl ring is identically substituted at both carbons ortho to
34 the point of phenyl ring's attachment to the remainder of the xanthene dye also exhibit desirable

1 fluorescent properties. Accordingly, in another embodiment, R^{11} and R^{15} are identical. Once
 2 again, the remaining carbons on the phenyl need not be substituted and, if substituted, the
 3 substituents may, independently, be the same or different when compared to R^{11} and R^{15} . In one
 4 embodiment, any substituents on the lower phenyl ring are identical.

5 Symmetry appears to be an important factor in selecting optimal C9 phenyl rings. In this
 6 regard, the symmetry is relative to an imaginary axis running from the lower phenyl ring's point
 7 of attachment (*i.e.*, the 10-carbon) to the remainder of the xanthene dye (*i.e.*, the 9-carbon)
 8 through a point *para* to the attachment (*i.e.*, the 13-carbon). Accordingly, in one embodiment,
 9 R^{11} and R^{15} are identical and the remainder of R^{12} , R^{13} and R^{14} are, identically, either hydrogen
 10 or a substituent different from R^{11} and R^{15} . In another embodiment R^{11} , R^{13} , and R^{15} are
 11 identical and the remainder of R^{12} and R^{14} are, identically, either hydrogen or a substituent
 12 different from R^{11} , R^{13} and R^{15} . In yet another embodiment, R^{11} , R^{12} , R^{14} and R^{15} are identical
 13 and R^{13} is either hydrogen or a substituent different from R^{11} , R^{12} , R^{14} and R^{15} . In still another
 14 embodiment, R^{11} , R^{12} , R^{13} , R^{14} and R^{15} are all identical. Optimal lower phenyl rings include
 15 those where the ring exhibits one of the aforementioned symmetries and R^{11} and R^{15} are selected
 16 from the same alkoxy and/or halo and/or haloalkyl group.

17 For the purposes of illustration, the following non-limiting examples of symmetrical halo
 18 and alkoxy substituted lower phenyl rings are provided, wherein X represents any halo group
 19 and R^A represents a (C1 to C20) alkyl:





21

1 where R^{11} and R^{15} are selected from alkyl, heteroalkyl, alkoxy, halo, haloalkyl, amino,
2 alkylthio, cyano, isocyano, cyanato, mercaptocyanato, nitro, and sulfinyl,
3 and the remainder of R^1 , R^2 , $R^{2'}$, $R^{3'}$, $R^{3''}$, R^4 , $R^{4'}$, R^5 , $R^{5'}$, $R^{6'}$, $R^{6''}$, R^7 , $R^{7'}$, R^8 , R^{12} , R^{13} ,
4 and R^{14} are, independently, selected from hydrogen and a substituent having no more than 40
5 atoms, typically no more than 25 atoms.

6 In one embodiment the phenyl xanthene dyes are rhodamines, namely, when A is an
7 amine group and B is an imminium group. In an alternative embodiment the phenyl xanthene
8 dyes are rhodols, namely, when A is an amine and B is a oxo group. In an alternative
9 embodiment, the phenyl xanthene dyes are fluoresceins, namely, when A is a hydroxyl group
10 and B is a carbonyl group.

11 The signal emitted by the phenyl xanthene dyes can be tuned by the selection of different
12 substituents. Especially beneficial substituents for tuning the phenyl xanthene dyes include R^{11} ,
13 R^{12} , R^{13} , R^{14} and R^{15} , with R^{11} and R^{15} being exceptionally effective for tuning the dyes. The
14 R^2 , $R^{2'}$, R^4 , $R^{4'}$, $R^{5'}$, R^5 , $R^{7'}$ and R^7 substituents are also beneficial toward tuning the spectral
15 properties of the dyes.

16 Usually, R^1 and R^8 are not, simultaneously, pendant or fused benzo, naphtho or
17 polycyclic arylene rings. The simultaneous presence of two relatively rigid aromatic
18 substituents immediately next to the 9-carbon phenyl may generate steric hinderances.

19 Symmetry can not only be an important factor in selecting optimal C9 phenyl rings, it
20 can also be a factor in selecting optimal fluorescein, rhodol and rhodamine type upper rings, as
21 well as a factor in selecting optimal phenyl xanthene dyes overall. Accordingly, it is desirable,
22 but not necessary, for the phenyl xanthene dyes to have identical $R^{3'}$ and $R^{6'}$ substituents (if
23 present) and/or identical $R^{3''}$ and $R^{6''}$ substituents (if present) and/or identical R^4 and R^5
24 substituents. Similarly, it is desirable, but not necessary, for the dyes to have identical R^1 and R^8
25 substituents and/or identical R^2 and R^7 substituents. Similarly, as already stated, it is often
26 desirable for R^{11} and R^{15} , as well as R^{12} and R^{14} , to be identical. The presence of one or more,
27 and especially all, of these symmetries facilitates the production of a strong signal.

28 In one embodiment, the phenyl xanthene dyes comprise core structure (I) and,
29 additionally, the substituents therein are defined as follows:

30 A is selected from $-OH$ and $-NR^{3'}R^{3''}$;

31 B is selected from $=O$ and $=N^{\oplus}R^{6'}R^{6''}$;

32 R^1 is selected from hydrogen, R^a , (C1-C20) alkyl or heteroalkyl optionally
33 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or

1 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
2 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
3 more of the same or different R^a or suitable R^b groups, or, alternatively, R¹ may be taken
4 together with R² form part of a benzo, naphtho or polycyclic aryleno group which is
5 optionally substituted with one or more of the same or different R^a or suitable R^b groups;

6 R² is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
7 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
8 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
9 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
10 more of the same or different R^a or suitable R^b groups, or, alternatively, R² may be taken
11 together with R¹ to form part of a benzo, naphtho or polycyclic aryleno group which is
12 optionally substituted with one or more of the same or different R^a or suitable R^b groups,
13 or, alternatively, when A is -NR^{3'}R^{3''}, R² may be taken together with R^{3'} to form a 5- or
14 6-membered ring which is optionally substituted with one or more of the same or
15 different R^a or suitable R^b groups;

16 R^{3'}, when present, is selected from hydrogen, (C1-C20) alkyl or heteroalkyl
17 optionally substituted with one or more of the same or different R^b groups, (C5-C20) aryl
18 or heteroaryl optionally substituted with one or more of the same or different R^a or
19 suitable R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with
20 one or more of the same or different R^a or suitable R^b groups, or, alternatively, R^{3'} may
21 be taken together with R² to form a 5- or 6-membered ring which is optionally
22 substituted with one or more of the same or different R^a or suitable R^b groups;

23 R^{3''}, when present, is selected from (C1-C20) alkyl or heteroalkyl optionally
24 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
25 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
26 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
27 more of the same or different R^a or suitable R^b groups, or, alternatively, R^{3''} may be
28 taken together with R⁴ to form a 5- or 6-membered ring which is optionally substituted
29 with one or more of the same or different R^a or suitable R^b groups;

30 R⁴ is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
31 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
32 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
33 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
34 more of the same or different R^a or suitable R^b groups, or, alternatively, when B is

1 -NR^{3'}R^{3''}, R⁴ may be taken together with R^{3''} to form a 5- or 6-membered ring which is
2 optionally substituted with one or more of the same or different R^a or suitable R^b groups;

3 R⁵ is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
4 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
5 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
6 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
7 more of the same or different R^a or suitable R^b group or, alternatively, when B is

8 -NR^{6'}R^{6''}, R⁵ may be taken together with R^{6''} to form a 5- or 6-membered ring which is
9 optionally substituted with one or more of the same or different R^a or suitable R^b groups;

10 R^{6''}, when present, is selected from (C1-C20) alkyl or heteroalkyl optionally
11 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
12 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
13 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
14 more of the same or different R^a or suitable R^b groups or, alternatively R^{6''} may be taken
15 together with R⁵ to form a 5- or 6-membered ring which is optionally substituted with
16 one or more of the same or different R^a or suitable R^b groups;

17 R^{6'}, when present, is selected from hydrogen, (C1-C20) alkyl or heteroalkyl
18 optionally substituted with one or more of the same or different R^b groups, (C5-C20)
19 aryl or heteroaryl optionally substituted with one or more of the same or different R^a or
20 suitable R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with
21 one or more of the same or different R^a or suitable R^b groups, or, alternatively, R^{6'} may
22 be taken together with R⁷ to form a 5- or 6-membered ring optionally substituted with
23 one or more of the same or different R^a or suitable R^b groups;

24 R⁷ is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
25 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
26 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
27 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
28 more of the same or different R^a or R^b groups, or, alternatively, R⁷ may be taken
29 together with R⁸ to form part of a benzo, naphtho or polycyclic arylene group which is
30 optionally substituted with one or more of the same or different R^a or suitable R^b
31 groups, or, alternatively, when B is -NR^{6'}R^{6''}, R⁷ may be taken together with R^{6'} to
32 form a 5- or 6-membered ring optionally substituted with one or more of the same or
33 different R^a or suitable R^b groups;

1 R^8 is selected from hydrogen, R^x , (C1-C20) alkyl or heteroalkyl optionally
2 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
3 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
4 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
5 more of the same or different R^a or suitable R^b groups, or, alternatively, R^8 together
6 with R^7 may form part of a benzo, naphtho or polycyclic arylene group which is
7 optionally substituted with one or more of the same or different R^a or suitable R^b
8 groups;

9 R^{11} and R^{15} are each, independently of one another, selected from halo, (C1-C20)
10 alkyl, haloalkyl, $-OR^y$, $-SR^y$, $-SOR^y$, $-SO_2R^y$, and nitrile;

11 R^{12} , R^{13} and R^{14} are each, independently of one another, selected from hydrogen,
12 R^x , (C1-C20) alkyl or heteroalkyl optionally substituted with one or more of the same
13 or different R^b groups, (C5-C20) aryl or heteroaryl optionally substituted with one or
14 more of the same or different R^a or suitable R^b groups and (C6-C40) arylalkyl or
15 heteroarylalkyl optionally substituted with one or more of the same or different R^a or
16 suitable R^b groups;

17 R^x is selected from $-NR^cR^c$, $-OR^d$, $-SR^d$, halo, haloalkyl, $-CN$, $-NC$, $-OCN$, $-SCN$,
18 $-NO$, $-NO_2$, $-N_3$, $-S(O)R^d$, $-S(O)_2R^d$, $-S(O)_2OR^d$, $-S(O)NR^cR^c$, $-S(O)_2NR^cR^c$, $-OS(O)R^d$,
19 $-OS(O)_2R^d$, $-OS(O)_2OR^d$, $-OS(O)_2NR^cR^c$, $-C(O)R^d$, $-C(O)OR^d$, $-C(O)NR^cR^c$,
20 $-C(NH)NR^cR^c$, $-OC(O)R^d$, $-OC(O)OR^d$, $-OC(O)NR^cR^c$ and $-OC(NH)NR^cR^c$;

21 R^y is selected from (C1-C20) alkyls or heteroalkyls optionally substituted with
22 lipophilic substituents, (C5-C20) aryls or heteroaryls optionally substituted with
23 lipophilic substituents and (C2-C26) arylalkyl or heteroarylalkyls optionally substituted
24 with lipophilic substituents;

25 R^a is selected from hydrogen, (C1-C8) alkyl or heteroalkyl, (C5-C20) aryl or
26 heteroaryl and (C6-C28) arylalkyl or heteroarylalkyl;

27 R^b is selected from $-NR^cR^c$, $=O$, $-OR^d$, $=S$, $-SR^d$, $=NR^d$, $=NOR^d$, halo, haloalkyl, ,
28 $-CN$, $-NC$, $-OCN$, $-SCN$, $-NO$, $-NO_2$, $=N_2$, $-N_3$, $-S(O)R^d$, $-S(O)_2R^d$, $-S(O)_2OR^d$,
29 $-S(O)NR^cR^c$, $-S(O)_2NR^cR^c$, $-OS(O)R^d$, $-OS(O)_2R^d$, $-OS(O)_2OR^d$, $-OS(O)_2NR^cR^c$,
30 $-C(O)R^d$, $-C(O)OR^d$, $-C(O)NR^cR^c$, $-C(NH)NR^cR^c$, $-OC(O)R^d$, $-OC(O)OR^d$,
31 $-OC(O)NR^cR^c$ and $-OC(NH)NR^cR^c$;

32 each R^c is independently hydrogen or R^d , or, alternatively, each R^c is taken
33 together with the nitrogen atom to which it is bonded to form a 5 to 8-membered
34 saturated or unsaturated ring which may optionally include one or more of the same or

different additional heteroatoms and which may optionally be substituted with one or more of the same or different R^a or R^d groups;

each R^d is independently R^a or R^a substituted with one or more of the same or different R^a or R^e groups;

each R^e is selected from $-NR^aR^a$, $=O$, $-OR^a$, $=S$, $-SR^a$, $=NR^a$, $=NOR^a$, halo, haloalkyl, $-CN$, $-NC$, $-OCN$, $-SCN$, $-NO$, $-NO_2$, $=N_2$, $-N_3$, $-S(O)R^a$, $-S(O)_2R^a$, $-S(O)_2OR^a$, $-S(O)NR^aR^a$, $-S(O)_2NR^aR^a$, $-OS(O)R^a$, $-OS(O)_2R^a$, $-OS(O)_2OR^a$, $-OS(O)_2NR^aR^a$, $-C(O)R^a$, $-C(O)OR^a$, $-C(O)NR^aR^a$, $-C(NH)NR^aR^a$, $-OC(O)R^a$, $-OC(O)OR^a$, $-OC(O)NR^aR^a$ and $-OC(NH)NR^aR^a$.

In another embodiment, the phenyl xanthene dyes comprise core structure (II) and, additionally, the substituents therein are defined as follows:

A is selected from $-OH$ and $-NR^{3'}R^{3''}$;

B is selected from $=O$ and $=N^{\oplus}R^{6'}R^{6''}$;

R^1 , $R^{3''}$, R^5 , $R^{6''}$, $R^{6'}$, R^7 , R^8 , R^{11} , R^{12} , R^{13} , R^{14} , R^{15} , R^x , R^y , R^a , R^b , R^c , R^d , and R^e are as first defined with respect to core structure (I),

R^2 is selected from hydrogen, R^x , (C1-C20) alkyl or heteroalkyl optionally substituted with one or more of the same or different R^b groups, (C5-C20) aryl or heteroaryl optionally substituted with one or more of the same or different R^a or suitable R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or more of the same or different R^a or suitable R^b groups, or, alternatively, R^2 may be taken together with R^1 or $R^{2'}$ to form part of a benzo, naptho or polycyclic aryleno group which is optionally substituted with one or more of the same or different R^a or suitable R^b groups;

$R^{2'}$ is selected from hydrogen, R^x , (C1-C20) alkyl or heteroalkyl optionally substituted with one or more of the same or different R^b groups, (C5-C20) aryl or heteroaryl optionally substituted with one or more of the same or different R^a or suitable R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or more of the same or different R^a or suitable R^b groups, or, alternatively, $R^{2'}$ may be taken together with R^2 to form part of a benzo, naptho or polycyclic aryleno group which is optionally substituted with one or more of the same or different R^a or suitable R^b groups, or, alternatively, when A is $-NR^{3'}R^{3''}$, $R^{2'}$ may be taken together with $R^{3'}$ to form a 5- or 6-membered ring which is optionally substituted with one or more of the same or different R^a or suitable R^b groups;

1 R^{3'}, when present, is selected from hydrogen, (C1-C20) alkyl or heteroalkyl
2 optionally substituted with one or more of the same or different R^b groups, (C5-C20) aryl
3 or heteroaryl optionally substituted with one or more of the same or different R^a or
4 suitable R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with
5 one or more of the same or different R^a or suitable R^b groups, or, alternatively, R^{3'} may
6 be taken together with R^{2'} to form a 5- or 6-membered ring which is optionally
7 substituted with one or more of the same or different R^a or suitable R^b groups;

8 R⁴ is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
9 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
10 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
11 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
12 more of the same or different R^a or suitable R^b groups, or, alternatively, when A is
13 -NR^{3'}R^{3''}, R⁴ may be taken together with R^{3''} to form a 5- or 6-membered ring which is
14 optionally substituted with one or more of the same or different R^a or suitable R^b groups,
15 or, alternatively, R⁴ may be taken together with R^{4'} to form part of a benzo, naptho or
16 polycyclic aryleno group which is optionally substituted with one or more of the same or
17 different R^a or suitable R^b groups; and

18 R^{4'} is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
19 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
20 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
21 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
22 more of the same or different R^a or suitable R^b groups, or, alternatively, R^{4'} may be
23 taken together with R⁴ to form part of a benzo, naptho or polycyclic aryleno group which
24 is optionally substituted with one or more of the same or different R^a or suitable R^b
25 groups.

26 In another embodiment, the phenyl xanthene dyes comprise core structure (III) and,
27 additionally, the substituents therein are defined as follows:

28 A is selected from -OH and -NR^{3'}R^{3''};

29 B is selected from =O and =N[⊕]R^{6'}R^{6''};

30 R¹, R^{3''}, R^{6''}, R⁸, R¹¹, R¹², R¹³, R¹⁴, R¹⁵, R^x, R^y, R^a, R^b, R^c, R^d, and R^e are as first
31 defined with respect to core structure (I);

32 R², R^{2'}, R^{3'}, R⁴, and R^{4'} are as defined with respect to core structure (II);

1 R^{5'} is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
2 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
3 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
4 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
5 more of the same or different R^a or suitable R^b group or, alternatively R⁵ may be taken
6 together with R⁵ to form part of a benzo, naptho or polycyclic aryleno group which is
7 optionally substituted with one or more of the same or different R^a or suitable R^b groups.

8 R⁵ is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
9 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
10 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
11 R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with one or
12 more of the same or different R^a or suitable R^b group or, alternatively, when B is
13 -NR^{3'}R^{3''+}, R⁵ may be taken together with R^{6''} to form a 5- or 6-membered ring which is
14 optionally substituted with one or more of the same or different R^a or suitable R^b groups,
15 or, alternatively, R⁵ may be taken together with R^{5'} to form part of a benzo, naptho or
16 polycyclic aryleno group which is optionally substituted with one or more of the same or
17 different R^a or suitable R^b groups;

18 R^{6'}, when present, is selected from hydrogen, (C1-C20) alkyl or heteroalkyl
19 optionally substituted with one or more of the same or different R^b groups, (C5-C20) aryl
20 or heteroaryl optionally substituted with one or more of the same or different R^a or
21 suitable R^b groups and (C6-C40) arylalkyl or heteroarylalkyl optionally substituted with
22 one or more of the same or different R^a or suitable R^b groups, or, alternatively, R^{6'} may
23 be taken together with R^{7'} to form a 5- or 6-membered ring optionally substituted with
24 one or more of the same or different R^a or suitable R^b groups;

25 R^{7'} is selected from hydrogen, R^x, (C1-C20) alkyl or heteroalkyl optionally
26 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
27 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
28 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
29 more of the same or different R^a or R^b groups, or, alternatively, when B is -NR^{3'}R^{3''+}, R^{7'}
30 may be taken together with R^{6'} to form a 5- or 6-membered ring optionally substituted
31 with one or more of the same or different R^a or suitable R^b groups, or, alternatively, R^{7'}
32 may be taken together with R⁷ to form part of a benzo, naptho or polycyclic aryleno
33 group which is optionally substituted with one or more of the same or different R^a or
34 suitable R^b groups; and

1 R⁷ is selected from hydrogen, R^a, (C1-C20) alkyl or heteroalkyl optionally
2 substituted with one or more of the same or different R^b groups, (C5-C20) aryl or
3 heteroaryl optionally substituted with one or more of the same or different R^a or suitable
4 R^b groups and (C6-C40) arylalkyl or heteroaryl alkyl optionally substituted with one or
5 more of the same or different R^a or R^b groups, or, alternatively, R⁷ may be taken together
6 with R^{7'} or R⁸ to form part of a benzo, naphtho or polycyclic aryleno group which is
7 optionally substituted with one or more of the same or different R^a or suitable R^b groups.

8 The list of possible phenyl xanthene dyes is as endless as the variations that can exist in
9 the upper ring. However, preferred dyes are fluoresceins, rhodols and rhodamines that have
10 found most use in the industry, as modified to contain the C9 phenyl ring discussed above.
11 Along this line, the following fluoresceins, rhodols and rhodamines are noted: Rhodamine 101,
12 Rhodamine 110, Rhodamine 6G, TAMRA, ROX, HEX, NAN, FLAN, TET, JOE, and ZOE.
13 Those skilled in the art will be able to name many more commercially important fluoresceins,
14 rhodamines and rhodols. Any fluorescein, rhodamine or rhodol can be modified at the C9
15 position to contain the lower phenyl ring.

16 **5.4 Lipid Soluble Phenyl Xanthene Dyes**

17 In one embodiment, the phenyl xanthene dyes not only contain the new lower phenyl
18 ring but also contain sufficient lipophilic groups to make the phenyl xanthenes lipid soluble.
19 This is especially beneficial when the phenyl xanthene dyes are used, for example, to imbibe
20 hydrophobic polymeric particles that are useful in aqueous assays. Such embodiments are
21 described, for example, in copending application no. _____, filed concurrently
22 with this application, entitled: "Fluorescent Polymeric Materials Containing Lipid Soluble
23 Rhodamine Dyes," attorney docket no. 71905.5010, incorporated herein by reference.

24 Non-limiting examples of such polymeric particles include crosslinked and
25 uncrosslinked polystyrene particles and styrene-(meth) acrylic acid copolymers. An unlimited
26 variety of particles for use in assays are commercially available, including particles that are
27 functionalized and/or paramagnetic and/or conjugated with a biological reagents. For example,
28 Bangs Laboratories sells the following products: "plain (hydrophobic) polystyrene
29 microspheres" of various sizes (catalog codes PS02N, PS03N, PS04N, PS05N, PS06N, PS07N,
30 PS08N, PS00N); "carboxylate-modified microspheres" of various sizes (catalog codes PC02N,
31 PC03N, PC04N, PC05N, PC06N, PC07N, PC08N and PC00N); "amino-modified
32 microspheres" of various sizes (catalog codes PA02N, PA03N, PA04N, PA05N, PA06N, and
33 PA00N); "classical magnetic microspheres" having carboxylic or amino functionality (catalog
34 codes MC02N, MC03N, MC04N, MC05N, and MC00N); "encapsulated magnetic

1 microspheres” with carboxylic and amino surface groups (catalog codes ME01N, ME02N,
2 ME03N, and ME00N); and “protein-activated” or “protein-coated” microspheres (catalog codes
3 CM01N, CM02N, CM03N, CP01N, CP02N and CP03N). Similarly, Dynal sells Dynabeads®
4 which are uniform, superparamagnetic, monodisperse polymer beads that can either be uncoated
5 or precoated with specific ligands. Dynabeads® are available in three different sizes, namely, 1
6 µm (Dynabeads® MyOne™ Streptavidin), 2.8 µm (Dynabeads® M-280 and Dynabeads® M-
7 270) and 4.5 µm (Dynabeads® M-450 and Dynabeads® M-500).

8 In such embodiments, the degree of lipid solubility required for the phenyl xanthene dye
9 necessarily varies as a function of the polymer utilized, the aqueous solvent or solvent system
10 employed in the assay in which the polymeric particle is to be used, and the conditions (*e.g.*,
11 time, temperature, pressure, pH, etc.) under which the assay is run. Suitable degrees of lipid
12 solubility are easily determined by methods known in the art. For example, suitable lipid
13 solubility can be determined by a partition test wherein a known quantity of dye in organic
14 solvent is combined with the aqueous solvent or solvent system used in the assay. If a partition
15 results and, under the conditions used in the assay, there is no appreciable crossing by the dye
16 into the solvent or solvent system, then the dye is sufficiently lipid soluble. Put another way, the
17 lipid soluble phenyl xanthene dye should be sufficiently lipid soluble such that it is capable of
18 being imbibed into the polymer when dissolved in an organic solvent or solvent system and,
19 when the dyed polymer is subjected to the aqueous conditions of the assay, the dye should resist
20 leaching out of the polymer to any degree that significantly impacts the fluorescent signature of
21 the dye imbibed polymer or the results of the assay.

22 In those embodiments where the phenyl xanthene dyes are lipid soluble rhodamines, one
23 or both of the exocyclic amine and exocyclic imminium nitrogens are often substituted with a
24 lipophilic group designed to impart to the rhodamine lipophilic characteristics or properties.
25 Thus, useful dyes include rhodamines that comprise the C9 phenyl ring described above and
26 additionally comprise one or two lipophilic substituents at the exocyclic amine nitrogen and/or
27 one or two lipophilic substituents at the exocyclic imminium nitrogen. In one embodiment, both
28 the exocyclic amine nitrogen and the exocyclic imminium nitrogen are substituted with a
29 lipophilic group. In another embodiment, the exocyclic amine nitrogen and the exocyclic
30 imminium nitrogen are both substituted with two lipophilic groups. The lipophilic groups,
31 whether attached to the same or different exocyclic nitrogen, may be the same or different. In
32 one embodiment, the lipophilic groups on the exocyclic nitrogens are the same.

33 In those embodiments where the phenyl xanthene dyes are lipid soluble rhodols, the
34 exocyclic amine nitrogen is often substituted with a lipophilic group designed to impart to the

1 rhodol lipophilic characteristics or properties. Thus, useful dyes include rhodols that comprise
2 the C9 phenyl ring described above and also comprise one or two lipophilic substituents at the
3 exocyclic amine nitrogen. In one embodiment, the exocyclic amine nitrogen is substituted with
4 one lipophilic group. In another embodiment, the exocyclic amine nitrogen is substituted with
5 two lipophilic groups. If there are two lipophilic groups on the exocyclic amine nitrogen, the
6 lipophilic groups may be same or different. In one embodiment, there are two lipophilic groups
7 on the exocyclic amine nitrogen that are the same.

8 Lipid-soluble phenyl xanthenes may include lipophilic substituents at other positions, as
9 well. It is the net effect of the lipophilic substituents that determines whether the phenyl
10 xanthene dye is lipid soluble. This is especially true for fluoresceins which have no exocyclic
11 amine or imminium nitrogens.

12 Lipophilic substituents are groups that impart the resultant phenyl xanthene dye with
13 lipophilic characteristics or properties as denoted above. The nature of each lipophilic
14 substituent is not critical, as long as the resultant phenyl xanthene dye is lipid soluble. Non-
15 limiting examples of suitable lipophilic substituents include unsubstituted (C4-C20) alkyls, (C5-
16 C40) aryls, and (C6-C40) arylalkyls. Depending on the number of methylene and methine units
17 in the lipophilic substituent, the lipophilic substituent may also include pendant or internal polar
18 or hydrophilic groups. For example, a lipophilic substituent may include one or more internal
19 heteroatoms, such as one or more internal O, S, N or NH groups. As another example, a
20 lipophilic substituent may include one or more pendant polar or hydrophilic substituents, such as
21 one or more pendant halogen, -OH, -SH, -NH₂, -C(O)OH, -C(O)NH₂ or other polar or
22 hydrophilic groups. Thus, lipophilic substituents may also include substituted (C4-C20) alkyl,
23 substituted (C5-C40) aryls and substituted (C6-C40) arylalkyls, as well as substituted and
24 unsubstituted (C4-C20) heteroalkyl, substituted and unsubstituted (C5-C40) heteroaryls and
25 substituted and unsubstituted (C6-C40) arylalkyls. The number of internal or pendant polar or
26 hydrophilic groups that may be included in a lipophilic substituent will depend upon, among
27 other factors, the number of methylene or methine groups included in the lipophilic substituent
28 and the number of lipophilic substituents on the phenyl xanthene dye. The nature and number of
29 lipophilic groups necessary to make a phenyl xanthene lipid soluble can vary from molecule to
30 molecule, and will be apparent to those of skill in the art.

31 **5.5 Conjugatable Phenyl Xanthene Dyes**

32 Oftentimes, it is desirable to attach fluorescent dyes such as the phenyl xanthene dyes
33 described herein to substances such as solid supports, particles, and biological and non-
34 biological molecules (e.g., drugs, amino acids, peptides, polypeptides, proteins, nucleosides,

1 nucleotides, oligonucleotides, polynucleotides, carbohydrates, etc.) Thus, in one embodiment,
2 the various phenyl xanthene dyes described herein include one or more moieties suitable for
3 such attachment. Such moieties are expressed by the formula $-S-LG$ where S is a direct bond or
4 a spacing moiety and LG is a linking group capable of forming a linkage with the substance to
5 be conjugated.

6 The linking group LG may be any moiety capable of forming the linkage, which may be
7 covalent or non-covalent. For example, the linking group may be one member of a pair of
8 specific binding molecules that non-covalently bind one another, such as biotin and
9 avidin/streptavidin. Thus, in one embodiment, the linking group is biotin. Alternatively, the
10 linking group may be a functional group capable of forming a covalent linkage with a
11 "complementary" functional group, such as an electrophilic (or nucleophilic) group which is
12 capable of forming a covalent linkage with a complementary nucleophilic (or electrophilic)
13 group, although other groups may be used depending on the desired linking chemistry, as is well
14 known in the art. Non-limiting examples of suitable electrophilic linking groups include any
15 one or a combination of the following: amines/anilines, alcohols/phenols, thiols, hydrazines and
16 hydroxylamines. Non-limiting examples of suitable electrophilic linking groups include any one
17 or a combination of the following: activated esters such as pentafluorophenyl ester and NHS-
18 ester, acrylamides, acyl azides, acyl halides, acyl nitriles, aldehydes, ketones, alkyl halides, alkyl
19 sulfonates, anhydrides, aryl halides, aziridines, boronates, carboxylic acids, carbodiimides,
20 diazoalkenes, epoxides, haloacetamides, halotriazines, imido esters, isocyanates,
21 isothiocyanates, maleimides, phosphoamidites, silyl halides, sulfonate esters and sulfonyl esters.

22 The linking group may be attached directly to the phenyl xanthene dye or it may be
23 spaced away from the phenyl xanthene dye by way of spacing moiety "S." As will be
24 appreciated by skilled artisans, the nature and composition of the spacing moiety is not critical
25 and may depend upon the particular application. Thus, the spacing moiety may comprise
26 virtually any combination of atoms or groups commonly employed to space one molecule from
27 another. As a specific example, the spacing moiety may be selected from substituted or
28 unsubstituted alkylenes or heteroalkylenes, substituted or unsubstituted arylenes or
29 heteroarylenes, substituted or unsubstituted arylalkylenes or heteroarylalkylenes, or a
30 combination of such groups. In one embodiment, the spacing moiety is an unsubstituted
31 alkylene of the formula $-(CH_2)_n-$, where n is an integer ranging from 1 to 40, typically from 1 to
32 20 and more typically from 1 to 10. Other exemplary spacing moieties and linking groups are
33 described, for example, in U.S. Patent Nos. 4,439,356, 4,481,136, 5,188,934, 5,654,442,
34 5,863,727, 5,847,162, 6,229,055, 6,248,884 and 6,372,907.

1 The linking group, whether attached directly or spaced away *via* spacing moiety "S,"
2 may be attached to any available position of the phenyl xanthene dye. For example, the linking
3 group may be attached to any available position on the upper ring or the lower ring. In one
4 embodiment, the linking group -S-LG is attached to the C2, C4, C5, or C7 position of the upper
5 ring. In another embodiment, the linking group -S-LG is attached to the C12, C13 or C14
6 position of the lower ring.

7 In one embodiment, a phenyl xanthene suitable for covalent attachment comprises any of
8 the previously-described phenyl xanthenes wherein one or more of R^1 , R^2 , $R^{2'}$, R^4 , $R^{4'}$, R^5 , $R^{5'}$,
9 R^7 , $R^{7'}$, R^8 , R^{11} , R^{12} , R^{13} , R^{14} or R^{15} is a substituent of the formula -S-LG, where S is a direct
10 bond or a spacing moiety and LG represents a linking group. In a specific embodiment, one of
11 R^4 , R^5 , R^{12} , R^{13} or R^{14} is -S-LG.

12 5.6 Conjugated Phenyl Xanthene Dyes

13 The lipid-soluble rhodamine dyes may be linked to another substance. In this
14 embodiment, at least one substituent on the phenyl xanthene dye is $-S^1-LK-S^2-CS$. In this case,
15 CS represents the conjugated substance and S^1 , LK and S^2 form what is known in the art as a
16 "linker" – which embraces any functionality known in the art that attaches a dye to another
17 substance.

18 S^1 and S^2 are, independently of one another, a covalent bond or a spacing moiety. The
19 nature of the spacing moieties S^1 and S^2 may vary broadly. Illustrative spacing moieties
20 include those previously specified for the spacing moiety "S."

21 LK represents a linkage, which may be a bond or another type of linkage, such as a
22 linkage formed between a nucleophilic (or electrophilic) group and a complementary
23 electrophilic (or nucleophilic) group. In one embodiment, LK is selected from an ester, an
24 amide, a sulfonamide, a hydrazine, an imine, a maleimide, a sulfide, a disulfide, a carbamate and
25 a thiocarbamate linkage.

26 The linker will vary depending the identity of the conjugated substance. Illustrative
27 linkers are provided, for example, U.S. Patent Nos. 4,439,356, 4,481,136, 5,188,934, 5,654,442,
28 5,863,727, 5,847,162, 6,229,055, 6,248,884 and 6,372,907.

29 In one exemplary embodiment, a conjugated phenyl xanthene is any of the previously-
30 described phenyl xanthene in which one or more of R^1 , R^2 , $R^{2'}$, R^4 , $R^{4'}$, R^5 , $R^{5'}$, R^7 , $R^{7'}$, R^8 , R^{11} ,
31 R^{12} , R^{13} , R^{14} or R^{15} is a substituent of the formula $-S^1-LK-S^2-CS$. In a specific embodiment, one
32 of R^4 , R^5 , R^{12} , R^{13} or R^{14} is $-S^1-LK-S^2-CS$.

1 **5.7 Energy Transfer Dyes**

2 In another embodiment, the phenyl xanthene dye is part of an energy transfer ("ET")
3 network comprising, for example, from two to four dyes covalently attached to one another that
4 transfer energy to generate a longer Stoke's shift. In other words, the phenyl xanthene dye may
5 be part of series of dyes that are covalently attached to one another. One example of an ET
6 network would be a fluorescence resonance energy transfer ("FRET") dye. In this embodiment,
7 at least one substituent on the phenyl xanthene dye is selected from $-S^1-LK-S^2-D$, where S^1 , S^2
8 and LK are as previously defined and D is another dye in the network. In one embodiment, each
9 dye in the energy transfer network is within 5 to 100 Å of the neighboring dye or dyes in the
10 network to which it is covalently attached. In such embodiments, the phenyl xanthene dye can
11 be the donor, acceptor, or an intermediate dye in the network.

12 In one embodiment, an energy transfer dye comprises any of the previously-described
13 phenyl xanthenes in which one or more of R^1 , R^2 , $R^{2'}$, R^4 , $R^{4'}$, R^5 , $R^{5'}$, R^7 , $R^{7'}$, R^8 , R^{11} , R^{12} , R^{13} ,
14 R^{14} or R^{15} is a substituent of the formula $-S^1-LK-S^2-D$. In a specific embodiment, one of R^4 , R^5 ,
15 R^{12} , R^{13} or R^{14} is $-S^1-LK-S^2-D$.

16 The identity of donor or acceptor dye is not critical, so long as it can donate or accept
17 energy from or to the particular phenyl xanthene to which it is attached. Dyes that can act as
18 donor or acceptors for phenyl xanthenes are well-known, and include, for example, other
19 fluoresceins, rhodamines, and rhodols, as well as cyanines, phthalocyanine and squaraine dyes.
20 Any of these dyes, or another phenyl xanthene as described herein, may be used as the donor
21 dye or acceptor dye in an energy transfer dye comprising the phenyl xanthene. The ability to
22 select a suitable dye for a particular phenyl xanthene is within the routine skill in the art.

23 As will be appreciated by skilled artisans, the various substituents S^1 , LK and S of the
24 linker should be selected to position the lipid-soluble rhodamine and acceptor or donor dye in
25 close enough proximity to one another such that the dyes can undergo energy transfer, whether
26 *via* FRET or another mechanism.

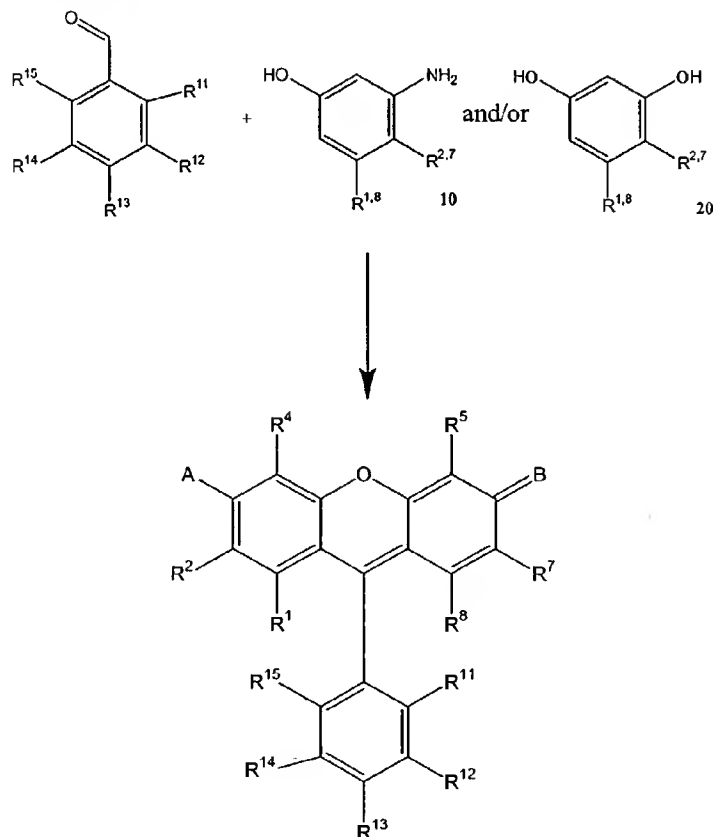
27 Suitable linkers are illustrated, for example, by U.S. Patent Nos. 5,800,996 and
28 5,863,727, issued to Lee et al., U.S. Patent No. 6,008,279, issued to Benson et al., and U.S.
29 Patent No. 5,654,419, issued to Mathies et al., all of which are hereby incorporated by reference.
30 Methods of synthesizing such energy transfer dyes, as well as suitable points of attachment for
31 covalently coupling the lipid-soluble rhodamine and acceptor or donor dye D to one another are
32 also described in these patents.

33 In one exemplary embodiment, an energy transfer dye of the formula $D^1-S^1-LK-S^2-D^2$,
34 where D^1 represents a phenyl xanthene dye, D represents another dye, and S^1 , LK, and S^2 are as

defined above, may be synthesized by reacting a phenyl xanthene of the formula D^1-S^1-LG , where LG represents a linking group, with a donor or acceptor dye of the formula D^2-S^2-LG' , where LG' represents a linking group which is complementary to linking group LG such that LG and LG' may react with one another to form linkage LK . As a specific embodiment, LG may be an activated ester such as an NHS-ester and LG' may be a primary amino group, such that reaction forms an amide linkage LK .

5.8 Method For Synthesizing Phenyl Xanthene Dyes

Phenyl xanthene dyes which include a C9-phenyl substitute which does not have an *ortho* carboxylate or sulfonate substituent may be prepared by the methods illustrated in figures as further described in the examples. In general, an *ortho* substituted benzaldehyde, which may or may not be further substituted, is reacted with either a substituted or unsubstituted 3-amino-1-hydroxybenzene or a substituted or unsubstituted 1,3-dihydroxybenzene or a mixture thereof. This is visually illustrated below:



where A is an amino or hydroxyl group, depending on whether it arises from compound **10** or **20** respectively, where B is an imminium or an oxo group, depending on whether it arises from

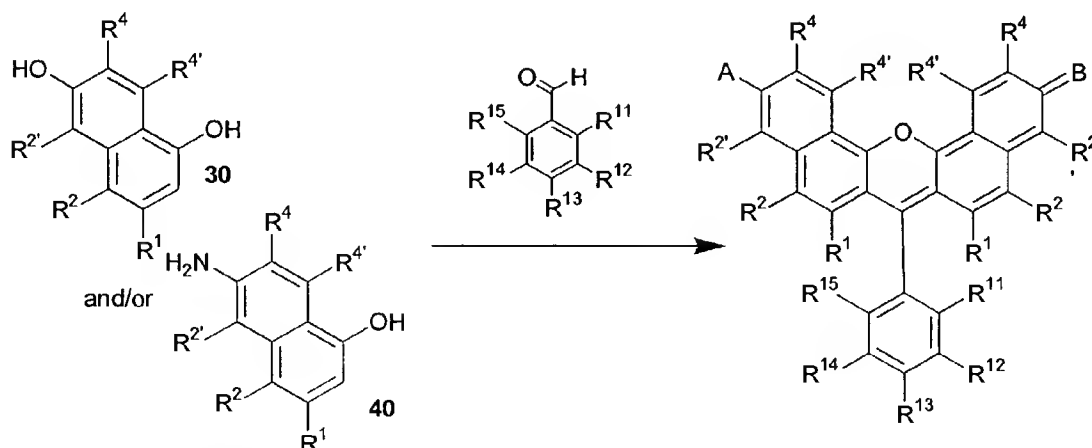
1 compound **10** or **20** respectively, and where the amine on compound **10** may be further
2 substituted with any desirable substituents for $R^{3'}$, $R^{3''}$, $R^{6'}$ and $R^{6''}$.

3 The reaction is carried out in solution, for example suspended in 1,2-dichlorobenzene.
4 The reaction is carried out under heat. Generally, a temperature ranging from 160 to 170 °C will
5 suffice. Preferably, a catalyst is employed. For reactions utilizing substituted or unsubstituted
6 3-amino-1-hydroxybenzene, lithium perchlorate is a good catalyst. For reactions employing 1,3-
7 dihydroxyphenol, toluene sulfonic acid is a good catalyst. Under these conditions, the reaction
8 takes about 60 minutes to complete.

9 Suitable aminophenol and benzaldehyde compounds are commercially available and
10 easily isolated or synthesized by one of skill in the art. For example, benzaldehyde can be made
11 by partial reduction of a benzoic acid, amide, or nitrile. Similarly, 3-amino-1-hydroxybenzene
12 can be manufactured by the reduction of nitrophenol as described in U.S. Patent No. 3,079,435.
13 Dihydroxy resorcinol and the like are commercially available from Aldrich Chemical
14 Company.

15

16 Extended phenyl xanthenes can be made by utilizing amino-hydroxy substituted
17 naphthalenes and/or dihydroxy substituted naphthalenes with, or in conjunction with the 3-
18 amino-1-hydroxybenzenes and 1,2-dihydroxybenzenes discussed above. This reaction is
19 visually illustrated below:



20

21 where A is an hydroxy or amino group, depending on whether it arises from compound **30** or **40**
22 respectively, where B is an oxo or an imminium group, depending on whether it arises from
23 compound **30** or **40** respectively, and where the amine on compound **40** may be further
24 substituted with any desirable substituents for $R^{3'}$, $R^{3''}$, $R^{6'}$ and $R^{6''}$.

25 **5.9 Improved Properties**

1 The phenyl xanthene dyes described herein, when excited by a light source, emit an
2 unusually strong spectral signal with low background noise. Lipid soluble embodiments of the
3 xanthene dyes are easily imbibed into, and retained by, hydrophobic polymeric particles, even in
4 the presence of water based solvents. Finally, the rhodamine dyes are highly photo and
5 chemically stable. In fact, some of the rhodamine dyes have photostabilities ten times that of
6 fluorescein and 100 times that of cyanine.

7 **5.10 Illustrative Uses**

8 The phenyl xanthene dyes of the instant invention have direct applications in a number
9 of technologies, including use as fluorescent labels in automated DNA sequencing,
10 oligonucleotide hybridization methods, detection of polymerase-chain reaction products,
11 immunoassays, and the like. For many applications, multiple dyes are employed, in
12 combination, to permit multiplex fluorescent detection.

13 The phenyl xanthene dyes can be used to sequence nucleic acids for example using the
14 Sanger method. the specifics of sequencing nucleic acids by the Sanger method are well-known
15 in the art and are not repeated here. For such sequencing applications, the phenyl xanthene dyes
16 described herein may be attached to the primer or to a terminating nucleotide, such as a 2,3'-
17 dideoxynucleotide triphosphate. Examples of various labeled primers, labeled terminating
18 nucleotides and methods of using such labeled primers and terminating nucleotides in
19 sequencing and other applications are described in U.S. Patent Nos. 5,188,934, 5,366,860,
20 5,654,442, 5,800,996, 5,840,999, 5,847,162, 5,863,727, 5,936,087, 6,008,379, 6,248,884 and
21 6,372,907, the disclosures of which are incorporated herein by reference. The xanthene dyes
22 described herein may be completed to similar primers and terminating nucleotides and used in
23 an analogous manner.

24 Alternatively, the phenyl xanthene dyes can be imbibed into particles used in the passive
25 or covalent coupling of analytes. In a particularly preferred aspect of the invention, a mixture of
26 lipid soluble phenyl xanthenes and, optionally additional dyes, are internally incorporated,
27 simultaneously or sequentially, into polymeric microparticles to give the microparticles a unique
28 spectral signature or "bar code." A number of particle populations are created, each
29 characterized by a different spectral bar code. The particles can then be activated or otherwise
30 modified so that they have a specific reactivity with one or more analytes in a clinical or test
31 sample. Thus, the spectral bar code in each particle population corresponds to a different known
32 reactivity. The particle populations can then be blended in a specified ratio to form a
33 multicolored particle mixture which is then contacted with the analyte. Imbibed bead mixtures

1 may contain hundreds to thousands of fluorescent dye molecules which greatly increases the
2 sensitivity of assays employing bead labels in comparison to single dye assays.

3 To achieve truly multiplexed analysis of a plurality of analytes in a single sample, some
4 sort of additional marker is necessary to show that a positive event has occurred on a particle.
5 This additional marker can be many things, for example, it can be a molecule, such as biotin,
6 which is detectable by its interaction with another compound, in this example streptavidin.
7 Alternatively, the additional marker can be second fluorescent signal, e.g., a green fluorescent
8 label. The marker is often provided by a labeling reagent which is also capable of binding to the
9 analyte of interest.

10 **5.11 Inherent Limitations In Structures**

11 Those skilled in the art will appreciate that many of the phenyl xanthene dye compounds
12 described in the various structures herein may exhibit the phenomena of tautomerism,
13 conformational isomerism, geometric isomerism and/or stereo isomerism. As the structures
14 presented in the specification and claims can represent only one tautomeric, conformational
15 isomeric, enantiomeric or geometric isomeric form, it should be understood that the invention
16 encompasses any tautomeric, conformational isomeric, enantiomeric and/or geometric isomeric
17 forms of the compounds that have one or more of the utilities described herein. As a specific
18 example, reference is made throughout the specification to the C3 amino and C6 iminium
19 substituents in rhodamines and rhodols. As this nomenclature corresponds to the illustrated
20 structures, which represent only one of several possible tautomeric forms (or resonance
21 structures) of the compounds, it will be understood that these references are for convenience
22 only and that any such references are not intended to limit the scope of the compounds described
23 herein.

24 Furthermore, those of skill in the art will recognize that the phenyl xanthene dyes of the
25 invention may exist in many different protonation states, depending on, among other things, the
26 pH of their environment. The structures provided herein depict the compounds in only one of
27 several possible protonation states. Accordingly, it will be understood that these structures are
28 illustrative only, and that the invention is not limited to any particular protonation state - any and
29 all protonated forms of the dyes are intended to fall within the scope of the invention.

30 As the phenyl xanthene dye compounds used in the invention may bear positive charges,
31 depending upon their physical state, they often have counterions associated therewith. The
32 identity or identities of any associated counterions is typically dictated by the synthesis and/or
33 isolation methods by which the compounds are obtained. Typical counterions include, but are
34 not limited to, halides, acetate, trifluoroacetate, any salt of a strong acid, and mixtures thereof. It

will be understood that the identity or identities of any associated counterions are not a critical feature of the invention and that the invention encompasses the use of dyes in association with any type of counter ion. Moreover, as the compounds can exist in a variety of different forms, the invention is intended to encompass not only forms of the dyes that are in association with counterions (*e.g.*, dry salts), but also forms that are not in association with counterions (*e.g.*, aqueous or organic solutions).

5.12 Incorporation By Reference

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. No admission is made that any reference cited in this specification is prior art.

6. EXAMPLES

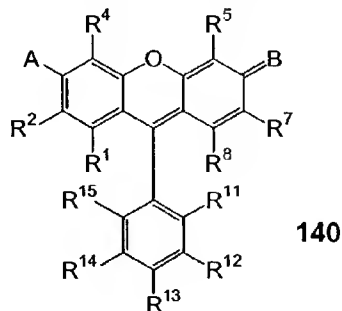
6.1 Overview of Synthesis for Fluorescein, Rhodol and Rhodamine Dyes

Exemplary phenyl xanthenes were synthesized from reactions of substituted and unsubstituted 1-hydroxy-3-aminobenzene derivatives and/or substituted and unsubstituted 1,3-dihydroxybenzene derivatives with phenyl aldehyde derivatives. Dye structures were verified by mass spectrometry.

6.2 Example 1

As illustrated in FIG. 1A, phenyl xanthenes containing symmetrically substituted upper rings were made by reacting one or more 3-amino-1-hydroxy-benzenes **120** (where $Y^{A,B}$ is an amine) and/or one or more 1,3-dihydroxy-benzene **120** (where $Y^{A,B}$ is a hydroxyl) with a phenyl aldehyde of general structure **130**. Reactants were suspended in a high boiling solvent, such as dichlorobenzene in the presence of a catalyst. Acid catalysts, such as toluene sulfonic acid, are used when $Y^{A,B}$ is a hydroxyl. Catalysts such as lithium perchlorate are used when $Y^{A,B}$ is an amine. Reactants were heated and stirred for 1 to 5 hours at a temperature from 130° to 155°C.

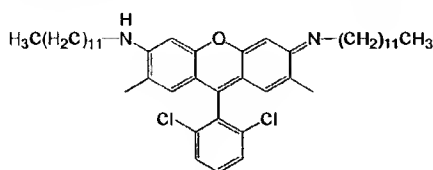
By this method, dyes corresponding to structure **140** were made:



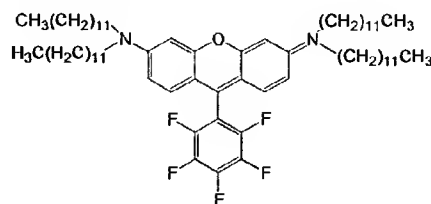
1 where $R^1=R^8$, $R^2=R^7$ and $R^4=R^5$. Symmetrically substituted rhodamines were produced by
 2 reacting a 3-amino-1-hydroxy-benzene with a benzaldehyde in the presence of lithium
 3 perchlorate. Symmetrically substituted fluoresceins were produced by reacting a 1,3-dihydroxy-
 4 benzene with a benzaldehyde in the presence of toluene sulfonic acid.

5 Phenyl xanthenes with non-symmetrically substituted upper rings can also be produced
 6 by employing multiple 3-amino-1-hydroxy-benzenes and/or multiple 1,3-dihydroxy-benzenes
 7 that contain different substituents.

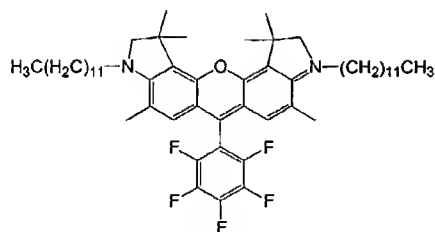
8 Dyes that were made by this procedure include the following:



Dye (3)



Dye (7)

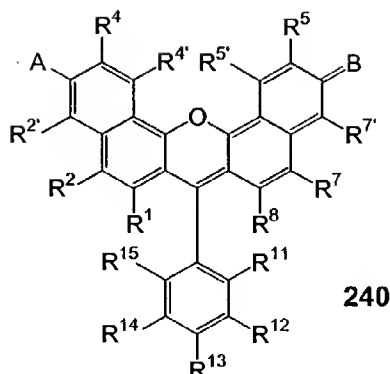


Dye (10)

9 6.3 Example 2

11 As illustrated in FIG. 2A, phenyl xanthenes with symmetrically substituted and
 12 symmetrically extended upper rings were made by reacting an amino-hydroxy-substituted
 13 naphthalene 220 (where $Y^{A,B}$ is an amine) and/or a dihydroxy substituted naphthalene 220
 14 (where $Y^{A,B}$ is a hydroxyl) with a phenyl aldehyde of general structure 130. Reactants were
 15 suspended in a high boiling solvent, such as dichlorobenzene, in the presence of a catalyst. Acid
 16 catalysts, such as toluene sulfonic acid, are used when $Y^{A,B}$ is a hydroxyl. Catalysts such as
 17 lithium perchlorate are used when $Y^{A,B}$ is an amine. Reactants were heated and stirred for 1 to 5
 18 hours at a temperature from 130° to 155°C.

19 By this method, symmetrically extended phenyl xanthene dyes corresponding to
 20 structure 240 were made:



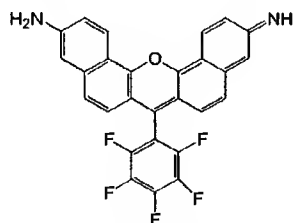
240

where $R^1=R^8$, $R^2=R^7$, $R^3=R^6$, $R^4=R^5$, and $R^4'=R^5'$. Symmetrically extended and symmetrically substituted rhodamines were produced by reacting an amino-hydroxy-substituted naphthalene with a benzaldehyde in the presence of a toluene sulfonic acid. Symmetrically extended and symmetrically substituted fluoresceins were produced by reacting a dihydroxy substituted naphthalene with a benzaldehyde in the presence of lithium perchlorate.

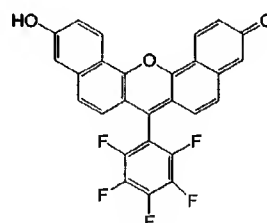
As illustrated in FIG. 2B, phenyl xanthenes with non-symmetrically extended upper rings can be synthesized by the same procedure if an additional reactant, namely a 3-amino-1-hydroxy-benzene (where $Y^{A,B}$ is an amine) and/or a 1,3-dihydroxy-benzene 120 (where $Y^{A,B}$ is a hydroxyl), is added to the reaction mixture.

Furthermore, phenyl xanthene dyes with non-symmetrically substituted upper rings can be synthesized by using a mixture of reactants with varying substituents as previously described.

Dyes that were made by this procedure include the following:



Dye (17)



Dye (19)

6.4 Example 3

Flouroscein dyes of structure 340 were synthesized in reactions of dihydroxy benzene derivatives 320 and benzoate ester derivatives 100 as outlined in Figure 3. Derivatives of 320 were synthesized by established procedures (patent Menchen et al. US 5,188,934, Benson et al. US 6,008,379, Upadya patent). As a general dye synthesis procedure 120 and benzoate ester 130 were suspended in neat methane sulfonic acid and the reaction heated at 130 °C for 3 hours. The reaction mixture was precipitated by pouring into ice, the solid precipitate collected by

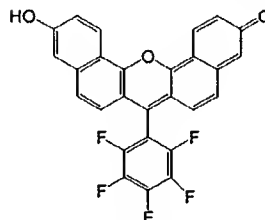
1 filtration, the crude dye **340** suspended in CH_2Cl_2 / MeOH / AcOH (95: 5: 0.5), and loaded unto
2 a silica gel column. The column was first eluted with CH_2Cl_2 / MeOH / AcOH (95: 5: 0.5) and
3 then CH_2Cl_2 / MeOH / AcOH (80: 20: 0.5). The fractions containing dye **340** were combined and
4 concentrated to a solid.

5 Alternatively, fluorescein dyes **340** were synthesized from reactions of benzaldehyde
6 derivatives **130** in dichlorobenzene with 10 equivalents of p-toluene sulfonic acid and the
7 reaction mixture was heated with stirring at 130°C for 3 hours.

8 **6.5 Example 4**

9 Extended fluorescein dyes of structure **440** were synthesized in reactions of 1,6-
10 dihydroxy naphthalene derivatives **320** and benzaldehyde derivatives as outlined in Figure 4.
11 Following the alternative general dye synthesis conditions described for **340**, 1,6-
12 dihydroxynaphthalene **420** and benzaldehyde derivatives **130** were suspended in dichlorobenzene
13 with 10 equivalents of p-toluene sulfonic acid and the reaction mixture was heated with stirring
14 at 130°C for 3 hours.

15 Following this general procedure, dye **19** below was produced from reactions of 1,6-
16 dihydroxynaphthalene **420** with compounds **130** where R11 - R15 = fluorine.



Dye (**19**)

18 **6.6 Example 5**

19 Rhodol dyes of general structure **540** were synthesized from reactions of equal
20 equivalents aminohydroxy benzene derivatives **120** or aminonaphthol derivatives **220**, dihydroxy
21 benzene derivatives **320** or dihydroxynaphthalene derivatives **420**, and phenyl aldehydes **130**,
22 following the general procedure described for synthesis of **140** outlined in Figure 5.

23 **7. SPIRIT OF THE INVENTION**

24 The invention now having been fully described, it will be apparent to one of ordinary
25 skill in the art that many changes and modifications can be made thereto without departing from
26 the spirit or scope of the invention. Accordingly, the scope of the invention is determined by the
27 claim limitations and any equivalents thereof as defined by law.